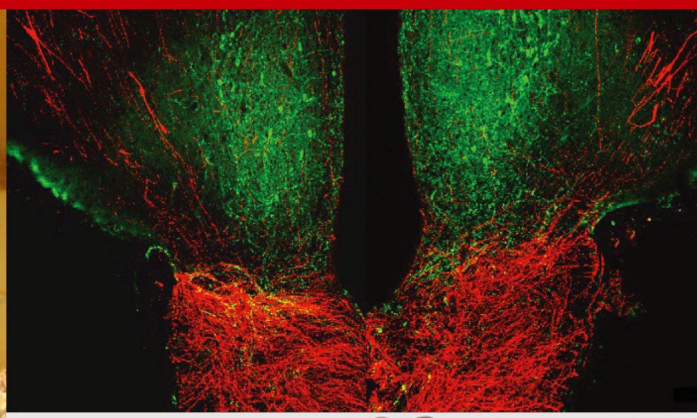
Knobil and Neill's

PHYSIOLOGY OF REPRODUCTION

FOURTH EDITION

VOLUME 2



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Pair bonded prairie vole family from Chapter 48 (J. Balthazart and L. Young). Photo by Todd H. Ahern

Relationship between gonadotropin releasing hormone GnRH (red) and kisspeptin neurons (green) in a coronal section of the monkey hypothalamus from Chapter 28 (A.J. Zeleznik and T.M. Plant).

Mouse uterus showing vascular permeability at the sites of implantation detected after an intravenous injection of a blue dye on day 8 of pregnancy. From Chapter 38 (S.K. Dey). Per Dey, acknowledgement/credit not needed.

Top back. Scanning electron micrograph of a bull sperm interacting with ciliated epithelium in the sperm storage reservoir of the oviduct (Chapter 5, Lefebvre et al., 1995).

Middle back from David Albertini. Confocal micrograph of a horse oocyte based on collaboration between Elaine Carnevale, Colorado State University and David Albertini and John Bromfield of the Kansas University Medical Center.

Lower back from Chapter 28 (A.J. Zeleznik and T.M. Plant). Pulsatile LH release from the pituitary and multi unit electrical activity in the mediobasal hypothalamus recorded from an intact (left panel) and ovariectomized (right panel) rhesus monkey.

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Introduction

This, the fourth edition of "Knobil and Neill's Physiology of Reproduction," is a major departure from its first three editions by having Editors in Chief not previously involved in managing the production of this work. Before his death in the year 2000, Ernst Knobil and I had made plans for the transition to an Editor in Chief who had been trained at the postdoctoral level in one of our laboratories. Dr Tony M. Plant was chosen from among several excellent candidates who had the requisite characteristics needed to continue production of a work that would have the same high standards we hoped to have maintained in the earlier editions. I first met Tony in the early 1970s while I was a young faculty member at Emory University in Atlanta, Georgia, and he was employed at the Georgia Mental Health Institute also located in Atlanta. I received a letter from Ernst Knobil inquiring about my impression of Tony, who had applied for a postdoctoral fellowship in Ernst's laboratory where I had trained as a fellow but had recently relocated to Emory. My reply was a strong endorsement of Tony, which was borne out in subsequent years. Tony Plant chose Dr Tony Zeleznik as a Co-Editor in Chief to share with him the demanding tasks of editorship requiring a broad base of information in the field and a high level of academic rigor. My perusal of the list of authors and their chapter titles suggests that the information presented in this edition will rival in quality that presented in the first three editions, i.e., the work is a modern synthesis of reproductive physiology of mammals at the molecular, cellular, and organismic levels. This is not surprising given the excellent past records of the two Editors in Chief.

Ernst Knobil and I believed strongly that any work claiming to be comprehensive and hewing to the highest principles of scholarship must include a summary of the founding and the founders of the field. Because Dr Roy O. Greep was one of the leading founders of the field, we requested that he provide that information together with an overview of the future of the field in his Foreword for the first edition in 1988. Thus, the original Foreword had the great strength of being written by one of the last surviving founders in the field, and thus avoided the greatest weakness of all historical analysis, namely that history is defined as what professional historians define it to be.

A new Foreword has been prepared by Dr M. Susan Smith to address, in part, Greep's predictions of progress that might occur in the field in the succeeding years after his Foreword first appeared. Susan is a prominent leader in reproductive physiology due, for example, to her Presidency of the Endocrine Society, Directorship of the Oregon National Primate Research Center, and maintaining an National Institutes of Health (NIH)-funded laboratory running for several decades. I am pleased that her fellowship training occurred in my laboratory at Emory University during which we published the most highly cited paper of my career, of which she was the senior author.

Jimmy D. Neill

Preface

Having had the fortune of being mentored by Ernst Knobil in the early stages of our careers and serving as assistant professors in his faculty in the Department of Physiology at the University of Pittsburgh School of Medicine, we were greatly honored by the invitation to serve as Editors in Chief for the 4th edition of this book that has come to be recognized by many as the "Bible of Reproduction." At the same time, we undertook the task with considerable trepidation, being fully aware of how difficult it would be to fill the shoes of Professors Knobil and Neill, who, with their utmost attention to scientific rigor, had, together or individually, guided the previous three editions.

Given the foregoing challenge, we began by evaluating whether the structure and content of the previous edition needed to be refocused. It quickly became obvious that this was indeed the case because of the rapidly growing use of "omics" approaches in most, if not all, areas of reproductive biology. In this regard, it is noteworthy that the first edition of this text was published in 1988, a year before the first use of the "gene knockout" approach in mice was described. Over the course of the 25-year history of this text, the ability to delete or modify individual genes has increasingly and significantly added to the classical armamentarium of organ ablation/hormone replacement paradigms previously available to investigators studying reproduction. In addition, the application of genetics/genomics to elucidate the etiology of human disease has increased in parallel to the use of transgenesis in experimental animals. Collectively, these powerful approaches have provided new, and often serendipitous, information on the regulation of reproduction. For example, our view of the neuroendocrine control of the hypothalamic–pituitary–gonadal axis is now completely dominated by kisspeptin. This hypothalamic peptide, which, in the context of reproduction, was unheard of before 2003, surfaced because mutations of its receptor, then known as GPR54, were identified in individuals with delayed puberty.

Accordingly, our authors were confronted with the need to integrate information gleaned from "classical" whole animal studies with results generated by contemporary "omics" approaches in order to provide a holistic overview of reproductive processes. In keeping with this approach, we encouraged authors to discuss the historical underpinnings of their respective fields and to identify the burning questions that remain to be addressed.

In this 4th edition, Volume 1 focuses on basic processes of male and female reproduction while the thrust of Volume 2 is on the physiological control systems that govern reproductive processes, including those regulating sexual behavior. A chapter on meiosis has been included for the first time, and discussions of epigenetic mechanisms that are emerging as important regulators of reproduction, as well as fetal origins of adult diseases, have been expanded. New chapters on spermatogonial stem cells and hormone signaling in the testis that provide contemporary views of the control of testicular function replace the more classical approach to the control of spermatogenesis that was presented in prior editions of the text. We have also expanded the discussion of the regulation of reproductive behavior, which, having moved far away from its origins in ethology, is now dominated by genomic, epigenetic, cellular, and molecular approaches to the understanding of sexual motivation, partner preference, and parental behavior. For those chapters that have been updated, we are confident that these new versions present the most current discussion of the subject material.

Such restructuring of this book would not have been possible without the enthusiasm and commitment of our section editors, to whom we are greatly indebted. A feature of the 3rd edition that has been maintained is the inclusion of the Foreword by Professor Roy O. Greep that was written for the 1st edition, published in 1988. As recognized by Professor Neill, the Editor in Chief of the previous edition, Greep's account of the future of research on the physiology of reproduction continues to remain remarkably relevant, now 26 years after it was written. We are pleased to include a second Foreword that was written by Dr M. Susan Smith, who worked with Jimmy Neill as a postdoctoral scholar and was also a member of Ernst Knobil's Department of Physiology at the University of Pittsburgh School of Medicine. Dr Smith's broad understanding of both whole animal and molecular approaches used in the study of reproductive processes makes her uniquely qualified to comment on how the study of reproduction has progressed since Greep's original Foreword in 1988, as well as to identify the challenges to be met in future years.

This 4th edition of *Knobil and Neill's Physiology of Reproduction* will be the last available in a print version. While our purpose here is not to debate the loss of hard copy, electronic publishing will enable, on the one hand, the addition of new chapters covering important emerging fields and, on the other, the replacement or revision of outdated chapters, both with relative ease. In this way, we anticipate that the *Physiology of Reproduction* will continue to be highly contemporary and comprehensive and therefore remain, for the future, the authoritative text of the field.

It is our expectation that this 4th edition of *Knobil and Neill's Physiology of Reproduction* will continue to kindle the importance of physiology in the understanding of reproductive processes and, in the words of Neill and Knobil in their preface to the first edition, be "useful to all serious students of reproductive physiology be they scientists, teachers or physicians."

Tony M. Plant
Anthony J. Zeleznik

Foreword* by Roy O. Greep

I am pleased and honored to have been asked to prepare the Foreword to this volume of work depicting the progress in research on the physiology of reproduction as well as the resulting gains in understanding made over the past few years. The expertise that is represented by the numerous contributors to this work is so impressive that I am humbled even to contemplate adding anything of note. It is only by virtue of having personally witnessed a very large segment of twentieth century research on reproduction that I am emboldened to reflect on the byways and the trailblazings that have brought this field to its present proud state of enlightenment with regard to the long sought-after means of controlling the procreative process in humankind. Clearly, there are many important and knotty problems yet to be resolved, but the pace of progress over the past several years has quickened to the extent that one is left in expectant wonderment as to where and when the next revolutionizing development will occur.

The experimental method of studying reproduction was initiated in 1849 with Berthold's discovery of a blood-borne activity that came from the testis and stimulated growth of distant organs such as the comb and wattles. In so doing he utilized one of the most fundamental means of demonstrating the function of an endocrine organ, namely, surgical removal to determine what deficiencies follow, coupled with implantation or transplantation to ascertain whether the deficiencies were repaired. At that time it was not possible to take the next step, namely, preparation of an active extract of the testes, because nothing was known about the nature of the bioactivity. Forty years later, Brown-Séquard claimed to have prepared an active extract of dog testes; however, as is well known, his enthusiastic claims for restoration of his own sexual activity at an advanced age were not substantiated. Actually, these simple means of studying reproductive physiology persisted well into the twentieth century, including the studies of such pioneering stalwarts as Marshall, Heape, Prenant, Bouin, Ancel, Loeb, Cushing, and Aschner. Observations otherwise were limited to cyclic and seasonal changes in sexual behavior among common laboratory and small domestic animals. This type of eyeball research remained in vogue through the early 1920s and overlapped the extension of visualization to the microscopic level. The latter revealed, for the first time, the precise timing of events in the ovarian cycle through microscopically observable cellular changes in the vaginal fluid. My point in mentioning these early studies is to emphasize that although the tools and techniques were inordinately primitive by present standards, the results established a firm base of knowledge on which to build.

The study of cyclic changes in the vaginal smear in rats and the findings of estrogenic activity in follicular fluid during the early 1920s led to an explosion of interest in the study of reproduction. The field was fortunate in attracting to its ranks a small band of exceedingly able biologists and biochemists who, in 1932, were to become authors of the classic first edition compendium, Sex and Internal Secretions, a volume overwhelmingly devoted to reproductive endocrinology. It was this landmark of progress that finally gave propriety to the study of reproduction and put it on a par with the study of other major bodily systems. Incredible as it may seem, it was only a decade earlier that a distinguished panel of the National Research Council had declared that sex research was not a fitting topic for scientific study.

Lest our pride in today's spectacular pace of progress unduly bedazzle the mind, it should not be overlooked that the developments recorded in the 10-year span from 1926 to 1936 may never be equaled. Among those monumental achievements, all of the native sex steroid hormones were brought to light, their structures were determined, their functions were defined, and they were made available in pure form for research and therapy. Similarly, all of the pituitary, placental, and urinary tropic hormones were identified, and their functions were defined. Like today's competition for priority rights, publicity, and potential financial gain, these earlier periods also were times for intense rivalries, but rarely with prospects for financial rewards. It would be difficult to overstate the boost that was given to basic and clinical research in reproduction as a result of the availability of estradiol-17β, testosterone, and progesterone in pure form and of known potency. The replacement of

*This Foreword by Roy O. Greep is reprinted from the 1988 (first) 1994 (second) and 2006 (third) editions of this work (E. Knobil and J. D. Neill, The Physiology of Reproduction. Raven Press, New York. Copyright Elsevier). This now-deceased author wrote a remarkably prescient account of the future of research in the field that remains as relevant today as it did in the first three editions.

homemade extracts and such elastic entities as rat units, mouse units, capon units, and so forth, with micrograms of pure hormone was revolutionizing and allowed the study of reproduction on a quantitative basis.

Prior to World War II the thrust of research on reproduction dealt predominantly with the steroid hormones. This was the heyday of steroid biochemistry. After World War II the emphasis shifted to the protein and peptide hormones, where it still remains strong. This prolonged and difficult effort yielded many biochemical triumphs. Most notable among these were the isolation of the pituitary, placental, and urinary gonadotropins, as well as the determination of their primary structure as glycoproteins comprised of two dissimilar and covalently bonded subunits, the isolation and synthesis of the gonadotropin-releasing hormone (GnRH) of hypothalamic origin, and the isolation and structural characterization of relaxin.

The availability of pure protein and polypeptide hormones made possible the production of hormone-specific antibodies as well as the application of immunological techniques to the study of reproduction. An outcome of great consequence was the development of radioimmunoassay as the new means of measuring all of the hormones relating to reproduction. The sensitivity of this new technique was so great that it made possible, for the first time, the measurement of all these hormones in the body fluids. It had the further distinct advantage of requiring such a small amount of fluid that the monitoring of blood levels of the hormones of reproduction could be done throughout an estrous or menstrual cycle by close serial sampling. This revealed still another and most unexpected finding, the pulsatile pattern of secretion.

Identifying the homeostatic mechanism(s) responsible for maintaining a steady state in various physiologic systems of the body has been fraught with many challenging problems, but these pale in comparison with the difficulties encountered in trying to elucidate the mechanisms maintaining a constantly changing system, a characteristic of the reproductive system of female mammals. The earliest piece of evidence suggested the existence of a "push–pull" mechanism that later came to be known as negative feedback. It was based on the demonstration that an estrogenic extract administered to immature rats would maintain the ovaries in an infantile state. This was quickly followed by conclusive evidence that estrogen acted to inhibit pituitary follicle-stimulating hormone (FSH) stimulation of follicular growth and maturation; however, the effect on luteinizing hormone (LH), ovulation, and luteinization remained unsettled. Gaps continued to exist in all proposed explanations of reproductive cycles. None of these explanations took into account the influence of photoperiodicity on seasonal breeders, nor did they account for the role of the stimulus of mating in nonspontaneous ovulators. Following the discovery of the hypothalamic control of pituitary function, estrogen was shown to exert its action on both the pituitary and the hypothalamus; however, the problem of accounting for cyclicity remained. Adding to the complexity, radioimmunoassay revealed an unexpectedly high level of blood estrogen just prior to ovulation, an event not in keeping with the negative feedback concept.

Finally, after many years of searching for a way out of this frustrating situation, a glimmer of light appeared at the end of this long dark tunnel—light that soon turned to brilliance. In 1969, Goding and associates found that the administration of large doses of estrogen to ewes at the time of estrus did not block, but instead entrained, ovulation. Shortly thereafter, in more elaborate examination of the relationship of blood estrogen levels and ovulation in rhesus monkeys in Knobil's laboratory, it was revealed that elevated estrogen levels preceded and appeared to trigger ovulation. On further examination, Knobil and colleagues found that when blood estrogen reached a critical level, the feedback mechanism switched from a negative to a positive, or stimulative, action. This utterly new finding greatly advanced our understanding of the endocrine mechanism governing reproductive cycles. There still remain, however, some uncertainties: Why does the switch in feedback action occur; to what extent and at what stage of the cycle does estrogen act at the level of the pituitary or the hypothalamus, or both; and lastly, what role, if any, do the ovarian peptides, especially inhibin, play in controlling reproductive cycles?

The progress of research on reproduction has been chronicled in numerous review articles by individual authors. Many have appeared in Recent Progress in Hormone Research, Volumes 1–42. Other major sources include the multiple editions of such titles as: Marshall's Physiology of Reproduction, Fourth Edition (1990); Sex and Internal Secretions, whose third and last edition was issued in 1961; two volumes on the Female Reproductive System (1973), and one on the Male Reproductive System (1975) in Section 7 of the Handbook of Physiology, published by the American Physiological Society; and four serial volumes on reproductive physiology in the International Review of Physiology, the last one being issued in 1983. The present volume will provide comprehensive coverage and meet the current needs of the field of reproductive physiology, a field that is rapidly gathering momentum from the application of new and highly sophisticated tools and techniques.

In viewing the vast literature dealing with research on the male and female reproductive systems and considering the rate at which it is accumulating, one might ask whether this staggering proliferation of books and articles is essential to progress; the answer is an emphatic "Yes!" The yardstick by which progress is measured

in this or any other field is not in the number of articles published or the amount of financial support but in improved understanding. Such gains are generally marked by sharp peaks at indeterminate intervals, separated by avalanches of incremental gains, as recorded in an ever-growing list of journals. The point to remember is that without this persistent chipping away at a major problem there would be no solutions and no quantum leaps forward. In research very little comes from out of the blue. Part of the driving force in research is its adventuresome nature and ever-present possibility that one's efforts will pay off in an important manner. It may not be entirely fair, but in research (as in most human activities), the spoils go to the victor in the form of kudos, prizes, awards, public attention, and, increasingly in the present technological age, monetary gains—sometimes of great magnitude. What effect this latter may have, if any, on the long-cherished sanctity of science has not been determined, but it has become a matter of concern.

This volume bears the title The Physiology of Reproduction. Physiology, by traditional consensus, is that branch of science, which studies the functions of a living organism or any of its parts and includes the basic underlying processes. It will be understood that most of the studies reviewed here will be based more on holistic research than on research at the submicroscopic or molecular level. It is unfortunate that the excitement generated by recent fantastic advances in molecular biology and development has tended to downgrade the value of whole-animal research, and physiology in particular is sometimes looked upon as passé. Actually, the two categories of research are complementary, and both are essential for maximum advancement of knowledge. Whole-animal research cannot become outdated because it is the quintessence of biological relevance and the means by which molecular findings must ultimately be evaluated.

In the same vein, no one immersed in reproductive endocrinology can be unaware of the current tendency to regard research at the molecular level as representative of exceptional scientific talent. This is a common consequence of the opening of a new arena of investigation. I recall an incident that happened at a scientific meeting back in the 1930s. The first three papers in a session chaired by an eminent embryologist were on endocrine topics—mine was the third. That being ended, the chairman took pains to assure the audience that the meeting could now turn to considerations of more fundamental nature. One of the other three papers was given by Herbert M. Evans, who bristled noticeably but held his fire. There was also an earlier period when one either worked on steroid biochemistry or something of lesser appeal like biology. Anyone who remembers the 1950s will recall a flash in the pan ignited by cybernetics, a study of automatic control systems both neural and physical. The gurus of cybernetics captured the attention of the press and of audiences throughout the land, but eventually this obsession suffered the fate of other passing preoccupations. My own observation is that the closer one approaches the molecular level of research, the more one becomes dependent on highly sophisticated instrumentation to make the observations and to read out results that are often quite free of extraneous variables. Toward the obverse situation, one's dependence on an extensive background of experience and physiological increases as does the unavoidable complex of in vivo variables that must be taken into account. In either case we have today the availability of far more diverse approaches to a given problem in any field of biomedical research than has ever existed before. In Berthold's day there was only one experimental method available; today's number is untold but is probably in the hundreds, perhaps thousands. This is an exceedingly promising situation and one to which investigators of all persuasions must adjust. Open minds will experience exhilaration over substantive achievements at any point on this observational spectrum.

One of the major factors influencing research on reproduction has been the availability of funds or lack thereof. Prior to the institution of federal funding (i.e., prior to the middle of the twentieth century), reproductive research was sparsely supported by university departmental funds, industry, small grants from the Committee for Research in Problems of Sex within the National Research Council, and some aid from the Rockefeller Foundation. The National Institutes of Health were slow in providing significant support of research on reproduction because of restrictions on the support of work related in any way to birth control. This occurred despite the simultaneous postwar baby boom. What kept research afloat during this critical period was major support by the Ford Foundation plus lesser contributions by other major foundations. It was not until the establishment in 1968 of the Center for Population Research in the NICHD that major governmental funding in this area became available, but the boost was short-lived. As a result of the imposition of fiscal restraints in the early 1970s, federal support dwindled and has remained at a minimal level ever since. Support from all sources is woefully incommensurate with the distressing expansion of the human population and the need for safe, effective, economical, and readily available means of limiting human fertility.

The physiology of reproduction is predominantly under hormonal control. The first essential step in studying reproduction was identification of the hormones involved and the functions they serve. This having been accomplished, efforts turned to a detailed analysis as to how hormones act within the body. During the 1980s there has been a rising tide of interest

in the binding of steroid, protein, or peptide hormones to receptors on specific target cells. Much effort is currently being directed toward the isolation and chemical characterization of these receptors. They are known to be composed of a protein or proteins, and some information has already been gained as to their partial or provisional structure. This, however, is only a preliminary step in the complex process whereby hormone action results in an end response such as growth, secretion of a target cell hormone, or altered behavior. The curtain has already been raised on the climatic and final chapter of the story on how hormones act. This involves linkage of the hormone–receptor complex with the nuclear genetic apparatus leading through a now well-defined series of processes to the manifestation of a physiological response in the living organism. Genes that bring about the expression of certain hormonal signals are being isolated, modified, transferred between species, and also inserted into bacteria where they direct the biosynthesis of specific hormones in large quantity. Thus genes are being manipulated in ways that raise the potential of altering the reproductive process. It is largely as a result of developments in endocrinology at the molecular level that bewildering possibilities loom on the horizons of reproductive research—they are within reach; they are science, not fiction; and they stagger the imagination.

It being granted that nothing succeeds like success, then the new edition of this highly successful two-volume compendium on The Physiology of Reproduction is destined for an illustrious fate. This second edition will maintain the same high standards of the first and again fulfill an existing need in a field that is experiencing rapid growth and exhilarating progress. Like the first edition, this one will provide a critical assessment of the state of the art in every aspect of research on the physiology of reproduction by eminent authorities.

In the years intervening between this edition and the last, notable changes have taken place in the study of reproduction. These stem largely from major advances in technology. Remarkable new instruments, techniques, and methods have enabled investigators to probe ever deeper into the interaction between hormones and genes, thereby eliciting in vivo responses. New parameters are being added to the target tissues of the classical reproductive hormones as revealed by the presence of receptor sites in tissues, the physiologic significance of which often remains tantalizingly obscure. Similarly, newly identified substances of endocrine or paracrine nature are being added to this domain of research with persisting frequency. Some of these substances—the endothelins, interleukins, activins, inhibins, and prorenin, to name a few—also exhibit a puzzling array of effects on extraneous tissues. Their study is being aided by the fact that their structure is known and, though rare, they are available.

Great strides are also being made in many other aspects of research on reproduction. Much work is being done on the structure of receptors and the loci of binding sites on segments of the folded gonadotropic molecules. A full-scale effort is underway seeking an elucidation of the neural mechanism underlying pulsatile secretion. Neuroendocrinologists are closing in on an elusive pulse generator located in the central nervous system. This looms as another landmark discovery in reproductive biology.

Research on reproduction is flourishing and the future appears bright. The taboos are gone. All aspects of the reproductive process are an open book. One area that has taken a quantum leap forward is the clinical application of an important body of relevant new knowledge gained in both basic and clinical spheres. Expanded opportunities have been opened by greatly improved diagnostic procedures, more effective treatment of disorders, and new methods of controlling fertility. Contributing greatly to this explosive development is the dissemination of information on reproductive matters to the lay public by the mass media. Concerned individuals have been made aware of the existing new means of manipulating the male and female reproductive systems for enhancement or inhibition of fertility. The joys and comforts that accrue respectively to these opposing modes of fertility control have enriched the lives of a grateful public. To that end I may note that it was by virtue of these frontier reproductive measures that my own progeny includes a new grandson and namesake.

Roy O. Greep

Foreword by M. Susan Smith

I am truly honored to write the Foreword for the 4th edition of this book; it is a humbling task to follow in the footsteps of Professor Roy O. Greep, whose Foreword is herein reprinted from the 1st, 2nd, and 3rd editions of this work. His remarkable account of the field of the physiology of reproduction was written 26 years ago, yet as you read it, you will develop an overwhelming appreciation for how fortunate we were to have Professor Greep as one of the foremost leaders in our field. It is especially gratifying for me to be associated with this great work, since my scientific lineage is part of the Greep/Knobil/Neill 'family', having trained with Professor Neill and served as a faculty member in Professor Knobil's Department of Physiology at the University of Pittsburgh.

Since the publication of the 3rd edition of this book in 2006, there continues to be remarkable progress in the physiology of reproduction, reflecting the use of advanced technologies, such as those spurred by contemporary genetic and genomic approaches. However, the challenge today is to understand how this genetic and molecular information is integrated into the manifestation of a physiological response. Greep's thoughts on this are still prescient today, "Actually, the two categories of research are complimentary…." "Whole animal research cannot become outdated because it is the quintessence of biological relevance and the means by which molecular findings must ultimately be evaluated."

Professor Greep issued a challenge in his Foreword: "Developments recorded in the ten-year span from 1926 to 1936 may never be equaled." I posit that the 10-year span from 2004 to 2014 was, at the very least, equally important, as we have made a paradigm shift in how we conduct our science. In the more traditional way of science, experiments focused on understanding control systems; this then led to the discovery of new molecules. Today, in contrast, the use of massively parallel sequencing (exome sequencing, ChiP-seq, and RNA-seq) allows us to identify all the players, even though we may have no idea of their function. We then "reverse engineer" results from these studies to discover where a particular molecule is produced, how its production is regulated, and what its function is. These sequencing techniques were used in the groundbreaking studies that signaled the important role of kisspeptin and neurokinin B in regulating the pituitary–gonadal axis; subsequent studies located their sites of production in the hypothalamus and identified their critical function in controlling GnRH neuronal activity. All of these sequencing techniques generate massive amounts of data and necessitate application of the extensive bioinformatics infrastructure that goes with managing large databases. Greep was right when he stated, "the closer one approaches the molecular level of research, the more one becomes dependent on highly sophisticated instrumentation to make observations and to read out results that are often quite free of extraneous variables." As an example of the power of these databases that are available to the scientific community, there is one that identifies all the transcribed genes in the ovary of the nonhuman primate and maps changes in their individual activities throughout the duration of a menstrual cycle and into early pregnancy. Not too long ago it would have been impossible to imagine being able to follow the activity of one gene through such a time course. Importantly, these databases can be used for comparative analyses among species and should provide enlightenment about the evolution of different approaches to reproduction.

The material in the chapters of this 4th edition reflects advances that have been made at all levels of the hypothalamic–pituitary–gonadal axis and the reproductive tract; many of these advances have been made possible by the new tools available to scientists. The most notable is the discovery of kisspeptin in the hypothalamus that fundamentally changed our concept of the control of GnRH neurons and has expanded our knowledge of the neural networks that govern reproductive function. Significant advances have also been made in our understanding of how G-protein coupled receptors function; such as the insight gained by the discovery of GnRH receptor misfolding that results in a loss of trafficking of the receptor to the plasma membrane and, thus, functionality, a deficit that can be overcome by artificial chaperones. New areas of research have come forth, such as oncofertility, a term coined to signify the restoration or maintenance of fertility in cancer patients whose gonadal function is diminished or lost due to the side effects of their radiation treatment or chemotherapy. Studies using new techniques, such as 3-dimensional culture of follicles in an extracellular matrix and differentiation of gamete stem cells, have provided new insights into the processes controlling folliculogenesis and gametogenesis, with translational

opportunities for promoting or controlling fertility. In addition, there is an explosion of interest in stem cell biology, a field that was rooted in reproductive research and the practice of in vitro fertilization. There is also a new appreciation for the role of the environment in fetal development and this has led to the birth of a new area of medicine known as DOHAD (Developmental Origins of Health and Disease). Maternal under- and overnutrition both lead to epigenetic changes in the fetus that can have long-lasting consequences in the adult. Similarly, exciting new research shows environmental influences on epigenetic modifications of DNA in sperm that may result in paternal transmission of disease risk.

When considering all of this new research, some caveats need to be kept in mind. There appear to be significant species differences in a number of reproductive processes, including puberty, ovarian cyclity, and parturition, yet current research focuses primarily on the transgenic mouse model because of the ability to manipulate the expression of specific genes. Therefore, many of our current ideas about the control systems regulating reproductive processes are "mouse-centric" and may not be directly applicable to other species. This may create problems as we look to translate this information into new therapeutics for use in human and veterinary medicine.

What new advances can we look forward to in the near future? New technologies, such as optogenetic/pharmacogenetic tools, designer receptors, and advanced imaging, will likely contribute to the making of another "best decade" from 2014 to 2024. But it will be important to develop better methods, with increased specificity and sensitivity, for measuring hormones and other substances in the blood. There are also growing concerns over the use of sex steroids in clinical therapies. As a result, there is considerable interest in developing "nonhormonal", i.e., nonsteroidal, selective therapies that act at the local or intracellular levels.

Future studies must also focus on the still many critical unanswered questions in the physiology of reproduction. Just in the area of neural regulation of reproduction alone, there are numerous examples. Many external signals modulate reproductive function, such as stress, endocrine disrupters, diet, photoperiod, and pheromones. Yet we still do not know how these external signals are transmitted to directly alter kisspeptin or GnRH neuronal activity and thus, reproductive function. Perhaps, Greep's assertion that "The physiology of reproduction is predominantly under hormonal control," needs to be expanded to include nonhormonal factors that are important modulators. Another mystery is what heralds the onset of puberty. Detailed information is known about the regulation of kisspeptin gene expression and its upregulation at the time of puberty, but the specific signals that bring about these epigenetic changes are still completely unknown. Similarly, although neuroendocrinologists are closing in on the GnRH pulse generator located in the central nervous system, the actual mechanisms involved in pulse generation remain elusive. Greep recognized that "This looms as another landmark discovery in reproductive biology." The processes involved in the positive feedback effects of estrogen on gonadotropin secretion also remain a mystery. With the current recognition that there are likely species differences in these processes, negative and positive feedback of estrogen might be achieved through two different populations of kisspeptin neurons (rodent model) or two different populations of GnRH neurons (nonhuman primate model).

While the future for research in reproduction appears bright, with more landmark discoveries in the making, there is also cause for concern. Funding for basic research in reproduction at the federal level is declining, as the emphasis shifts to translational research relevant to human health and disease. In the bench-to-bedside continuum of research, it is critical to keep "bench" in the equation. There are still many areas in reproduction where we do not understand the basic underlying controlling mechanisms, making it difficult to devise therapeutics. Studies of various species will also be harder to support. This is regrettable, since comparative studies have revealed differences in how reproduction is regulated, and this collective information may be critical to solving long sought after questions, such as, what constitutes the GnRH pulse generator, how is puberty initiated, how is parturition initiated, and what is the impact of environmental factors on germ cells. A good example of the value of studying various species is the seasonal regulation of reproduction that led to a focus on photoperiod and how time-of-day signals are transmitted to GnRH neurons. These studies were instrumental in advancing the field of chronobiology to the recognition today that all cells appear to have clock genes that regulate their function. It is also regrettable that there are still constraints on contraception research even though the link between the ability to control fertility and the economic development of a country is well established. Finally, in this age of "big data," it is well to remember that analysis of large databases can detect correlations, but it cannot determine whether the correlations are meaningful or provide information about causality or mechanisms. This is the province of basic research: to discover the critical information about control systems that then provides the underpinnings for translational research and the development of new human therapeutics.

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CHAPTER

26

Neuroendocrine Control of the Ovarian Cycle of the Rat

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INTRODUCTION

In all female mammals, fertility is maintained by hormonal signals conveyed between the brain, pituitary gland, and ovaries, functioning together as a hypothalamic–pituitary–ovarian hormone axis. Hypothalamic neurons in the basal forebrain secrete gonadotropin-releasing hormone (GnRH) into a hypothalamic–hypophysial portal vessel system to stimulate release of the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which circulate to regulate ovarian functions, including steroidogenesis, folliculogenesis, and ovulation. Ovarian hormones in turn exert negative feedback effects within the brain and pituitary gland to maintain homeostatic control within the axis. In addition, they convey special positive feedback actions that evoke a gonadotropin surge that triggers ovulation in mature ovarian follicles. These feedforward and feedback signals orchestrate the sequence of events that unfold during the course of each ovulatory cycle in spontaneously ovulating animals. More so than any other experimental animal, the laboratory rat has been studied to obtain a fundamental understanding of these physiological mechanisms that govern ovarian cyclicity.

The laboratory rat has also served as a reference point for the elucidation of species-specific aspects of ovarian cyclicity and its control by neuroendocrine systems (Chapter 33). Indeed, much of what is known about the control of the ovarian cycle of many spontaneously ovulating, nonseasonal estrual mammals is based on our knowledge of the controls of the estrous cycle of the rat. However, unlike the ovarian cycles of other spontaneous ovulating mammals (sheep and primates; also described in this section of the book), the rat as well as the mouse exhibit a cycle that is characterized by a brief luteal phase. Moreover, the events of the ovarian cycle of the rat are tightly coupled to the 24-h light–dark cycle. That is, the lighting periodicity plays a dominant role in the incidence and duration of the stages of the ovarian cycle.

This chapter will present a detailed description of the estrous cycle of the rat, with emphasis on the neuroendocrine control mechanisms that govern the ovarian cycle. The reader of this edition will note the increased frequency with which studies of genetically modified mice are cited to support the basic physiological principles that govern the ovarian cycle in both species. However, the reader should be aware that the neuroendocrine mechanisms regulating ovarian cyclicity in the two species overlap to a great degree, but are not identical. Rats and mice both exhibit spontaneous 4- to 5-day estrous cycles characterized by similar, if not identical, patterns of hormone secretion and cyclic ovarian function. Olfactory cues, however, can play a much more prominent role in regulating cyclicity in mice, while photic cues more robustly dictate the timing of ovulation in rats. These differences notwithstanding, I will remain true to the original intent of this chapter—to review the neuroendocrine regulation of the ovarian cycle of the rat—while incorporating into the discussion the newer findings obtained using the power of mouse genetics, specifically those observations that clearly support and further elucidate neuroendocrine principles common to both species, which are informative to the understanding of these processes in other species.

An understanding of the neuroendocrine regulation of ovarian cyclicity began with the recognition that the brain releases factors that direct the secretion of anterior pituitary hormones and that these, in turn, circulate to regulate ovarian function. Harris demonstrated in 1937 that electrical stimulation of the hypothalamus induces

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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ovulation in the rabbit.[1] In the same decade, the existence of a specialized hypothalamo-hypophysial portal vasculature was revealed in which blood flows from a primary capillary bed in the median eminence (ME) through long portal vessels that drain toward sinusoids in the anterior pituitary.[2,3] The description of the hypothalamo-hypophysial portal vessels led Harris to propose that they function to convey signals that mediate the ovulatory response to electrical stimulation of the hypothalamus.[4] Harris supported this view by demonstrating that restoration of reproductive function in stalk-transected rats was proportional to the degree of portal vessel regeneration.[5,6] Harris and Jacobsohn[7] also transplanted pituitary glands from neonatal donor rats to various neural and nonneural sites in hypophysectomized recipient rats. Only when the gland was transplanted to just beneath the ME and vascularized from the ME were full reproductive and other pituitary functions restored. With these and other observations, it was finally appreciated that hypothalamic control of reproductive function was due to a neurovascular link between the hypothalamus and pituitary gland.

The chemical nature of the molecules synthesized by neurons and transported by this specialized vascular link thus became the subject of studies attempting to identify the hypothalamic releasing factors that control the synthesis and secretion of the major anterior pituitary hormones,[8,9] including LH and FSH. An activity that is capable of releasing LH was found in extracts of ME by both McCann[10] and Harris[11] working independently. McCann later suggested that a luteinizing hormone releasing hormone (LHRH), now more commonly known as GnRH, exists in the hypothalamus, which activated the release of LH.[12] In 1971, Schally's laboratory and that of Guilleman successfully isolated the decapeptide from extracts of porcine and ovine hypothalamus, respectively, and identified its chemical structure.[13,14] With the synthesis of the decapeptide, antibodies were produced for purposes of radioimmunoassay,[15] and immunohistochemical localization of GnRH-neurons within the brain.[16,17] The peptide fulfilled all the expectations as a neurohormone: identification in neurons, release into portal blood, and activity correlated with release of LH and FSH into peripheral plasma. Decades of research have since established the primacy of GnRH as the neural effector of reproductive function in both sexes and in all mammalian species. In much of this chapter, the special importance of GnRH in the neuroendocrine regulation of the estrous cycle of the rat is considered.

The chapter begins with an overview of the estrous cycle and proceeds to build a hierarchical understanding of ovarian cyclicity by focusing in turn on ovarian, pituitary, and then neuroendocrine physiological controls. I consider the cyclic physiological processes that occur within the ovaries, the pituitary hormone secretions that control these cyclic ovarian phenomena, and then the neural mechanisms that register environmental and endocrine signals and transduce them into the GnRH release patterns that ultimately drive the pituitary and ovarian events of the ovarian cycle.

OVERVIEW OF THE ESTROUS CYCLE

The word *estrus* is a Latin adaptation of the Greek word *oistros* meaning gadfly, sting, or frenzy. This term was first used by Heape[18] to describe the "special period of sexual desire of the female" and distinguish it from rut in the male. Heape further described distinct stages of the cycle as it applied to mammals during the breeding season. He used the term *anestrus* to describe the nonbreeding season or period of rest in the female mammal when the ovaries and accessory reproductive organs are relatively quiescent and attempts of mating by the male are resisted. Heape also used the prefixes *pro-*, *di-*, and *met-* along with the suffix *-estrus* to describe the stages of the cycle between the periods of estrus during the sexual season. The first part of the cycle he termed *proestrus*, characterized as the time when an animal is "coming on heat." The next period, estrus, Heape described as "only at that time that the female is willing to receive the male, and fruitful coition rendered possible in most, if not all, mammals." In the absence of conception, estrus is succeeded by a short recovery period called metestrus, during which the estrus changes in the reproductive tract subside. The following period, diestrus, is of variable duration in different species and is analogous to the luteal phase observed in primates and sheep, but lasts only 1–2 days in the unmated rat or mouse before a return to proestrus and progression through the next cycle.

The laboratory rat is a nonseasonal, spontaneously ovulating, polyestrous mammal. That is, the ovarian cycle continues throughout the year (as opposed to ovarian cycles restricted to one season, as in many sheep). Ovulation is not dependent on overt nervous stimulation (as opposed to the requirement of the mating stimulus in rabbits). Ovulation occurs every 4–5 days throughout the year (the dog ovulates once in the spring and once in the fall). The mean length of approximately 2000 cycles in the rat was described by Long and Evans[19] to be 5.4 days with a range of 3–38 days. If cycles of greater than 8 days are excluded, the mean was 4.8 days, which compare favorably with 4.4 days of Blandau, Boling, and Young[20] and 4.5 days of Astwood.[21] The length of the individual phases of the estrous cycle based on vaginal smear pattern has also been described.[19,21–24] Proestrus lasts for 12–14 h; estrus, 25–27 h; metestrus, 6–8 h; and diestrus, 55–57 h.

The stages of the estrous cycle can be conveniently determined by monitoring, under low magnification, the cell types which appear in the vaginal smear. Proestrus is characterized by a predominance of nucleated epithelial cells. These cells are distinctly round, visibly nucleated and appear in clusters at the height of proestrus. Occasionally, a few cornified squamous epithelial cells may appear as well. On the next day, estrus, the dominant cell is the cornified squamous epithelial cell. This cell is without an observable nucleus, contains a highly granular cytoplasm, and is irregularly shaped. These also appear in large groups. During metestrus (often called diestrus-1) and diestrus (sometimes called diestrus-2), the dominant cell type is the leukocyte, which appears along with a significant number of nucleated epithelial cells. The leukocytes are small with granular cytoplasm and, if examined under higher magnification, usually contain a vesiculated nucleus. Once again, the following day, proestrus, is characterized by the complete absence of leukocytes in the vaginal smear and the predominance of nucleated epithelial cells. The 5-day cycle may appear as an additional day of cornification (estrus) or an additional day of leukocyte infiltration (diestrus).

OVARIAN EVENTS DURING THE ESTROUS CYCLE

Oogenesis

The oocyte develops during fetal life through the prophase stage to the dictyate or "resting stage" of meiosis I by 4 days after birth[25] (Chapter 1 and 2). This stage is characterized by the presence of a distinct germinal vesicle or nucleus, the faint appearance of the diploid number of chromosomes ($n = 42$), and a distinct nucleolus. The oocyte remains arrested at this stage of meiosis until ovulation occurs (see below). The timing of the resumption of meiosis has been reported with respect to the onset of sexual receptivity,[26] the phases of "natural" or artificial illumination,[25] and the timing of the ovulation inducing release of LH.[27] The resumption of meiosis I consists of a reduction division and is controlled by the preovulatory surge of LH[28] (Chapter 1 and 2). That is, the chromosome number is reduced from the diploid content of 42 to the haploid number of 21. The first signal that meiosis I has been re-initiated is breakdown of the germinal vesicle, the disappearance of the nuclear membrane, and chromosome thickening. This occurs between 17:00 and 18:00 h on proestrus[25] or approximately 2–3 h after the beginning of the ovulation-inducing release of LH and 7–9 h before the expected time of ovulation.[27] During the first metaphase, the paired chromosomes are aligned along a metaphase plate. They are attached to their respective centromeres by spindle fibers. At anaphase I, the chromosomes begin separating, with half the chromosomal material ($n = 21$) lost by polar body extrusion during telophase I. Extrusion of the first polar body has been variously reported to be complete by 4–8 h after the beginning of sexual receptivity,[26,29,30] by 21:00 h proestrus,[25] or by 22:00 h on proestrus to 03:00 h estrus.[27]

Once the first polar body is extruded, ovulation occurs. This occurs by 8–10 h after the beginning of sexual receptivity,[26,30–32] 9–10 h after the resumption of meiosis I,[25] or by 03:00 to 05:00 h on estrus.[27] The newly ovulated egg found in the upper third of the oviduct is now haploid, with one polar body extruded and the remaining chromosomes aligned along a new metaphase plate. The tubal ovum, now in metaphase II, is available for fertilization by the haploid sperm. The second meiotic division proceeds if the ovum is fertilized (Chapter 1,2 and 4).

Folliculogenesis

The maturational process of the oocyte described above occurs in association with the growth of the ovarian follicle. The control of folliculogenesis is described in detail in Chapters 2 and 21, and is briefly discussed here. During late fetal and early postnatal development,[33] a single layer of epithelial cells becomes arranged around the oocyte in a uniform capsule. Such follicles, with a single layer of flattened epithelial cells surrounding the oocyte, are called primordial follicles. Follicular growth begins upon the activation of primordial follicles, an event associated with the transformation of the flattened epithelial cells surrounding the oocyte into cuboidal granulosa cells and is triggered, at least in the mouse, by the AKT/PI-3 kinase pathway (Chapter 21). The primordial follicles then become primary follicles (Figure 26.1). The granulosa cell layer proliferates, first forming a double, and finally a multilayered, cuboidal follicular epithelium. At this stage, the follicles are identified as secondary follicles. The growth of the follicle from the primary to the secondary stage is thought to occur independently of FSH and LH; rather, communication between the oocyte and the granulosa cells by members of the transforming growth factor-β (TGF-β) family is primarily responsible for this phase of follicular development (see Figure 21.1 in Chapter 21).

Under the influence of FSH, LH, and TGF-β family members, multilayered secondary follicles are transformed into vesicular follicles with the formation of multiple fluid-filled intracellular spaces. These spaces eventually enlarge and coalesce into a single larger cavity, the antrum. The structure is now termed a tertiary follicle, which upon further growth to the preovulatory stage is known as a Graafian follicle. The oocyte, together with its attached granulosa cells (which once occupied the center of the secondary follicle), now become eccentric and form a hillock, the cumulus oophorus. The granulosa cells surrounding the encapsulated oocyte form a ring known

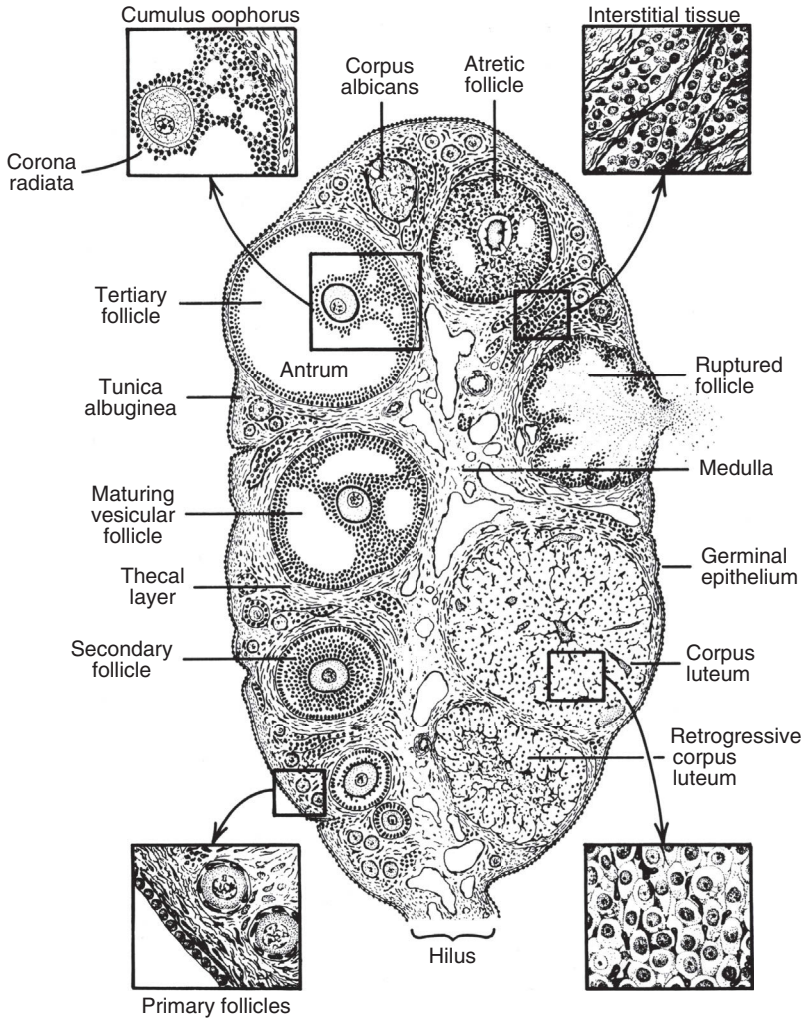

FIGURE 26.1 Composite stages of ovarian follicular development through ovulation, corpus luteum formation, and regression.

as the corona radiata. The fluid contents of the antrum, the liquor folliculi, increase in volume as ovulation approaches. The enlargement of the granulosa cell layer is accompanied by the development of an outer encapsulating sheath derived from the stroma. This constitutes the theca, which can be divided into a theca interna and theca externa. This division is first noted in the secondary follicle. The theca interna cells rapidly enlarge and assume a polygonal shape with vacuolated cytoplasm and vesicular nuclei. These cells are enmeshed in a reticular and fibrous network and contain a plexus of capillaries and lymphatics. This layer hypertrophies as proestrus approaches and is considered one of the major sites of steroid production during the cycle.[34,35] Indeed, owing to its glandular appearance, it has been referred to as the thecal gland.[36] The outermost follicular layer, the theca externa, contains contractile tissue, which is thought to play a role in ovulation.[37,38]

Very few of the follicles present on the ovary at the time of birth are destined to ovulate. Indeed, in the rat, Arai[39] calculated a decline in the total number of follicles from 35,000 at the time of birth to 11,000 at 20 days later. Thereafter, the rate of decline in the number of follicles decreases. This attrition of follicles, known as atresia, occurs throughout the reproductive lifespan. However, in contrast to primates, the supply of primordial follicles in rats and mice is not exhausted in aged animals. Rather, reproductive senescence in rats and mice is due to age-related alterations in the hypothalamic–pituitary axis (see Chapter 37).

Though some atresia of primordial and primary follicles occurs, atresia of secondary and vesicular follicles is most conspicuous during each cycle. The appearance of the atretic follicles differ depending upon the stage of the follicle at the time degeneration begins and the extent of the retrogressive changes at that time.[33] The questions of what determines which follicles ovulate and whether atresia begins in the oocyte or follicular epithelium have remained largely unanswered.[40] Also, the mechanism for maintenance of the viable follicle pool is not clear. Some

studies have suggested that adult mice have the capacity to generate new ova from adult germline stem cells originating from bone marrow and that transformation of these cells and incorporation into ovarian follicles can continuously replenish the ovarian follicle pool.[41] More recent studies have not provided support for adult neooogenesis as a replenishment mechanism,[42] and mathematical models for the kinetics of follicular development and atresia have reaffirmed that the classical model of a non-replenishable follicle pool adequately accounts for the observed follicle numbers throughout postnatal life.[42]

The first recognizable atretic changes are found in the epithelial cells lining the antral boundary of vesicular follicles and in the cells just external to the corona radiata of secondary follicles. Under conventional staining, the first sign of the process is a shrinking of the nucleus and condensation of the chromatin mass of the granulosa cells. This is known as pyknosis. In addition, the granulosa cell nucleus may rupture and the chromatin disintegrates into formless granules, which are extruded from the cell. This is known as karyorrhexis. In vesicular follicles, these nuclear fragments and pyknotic cells tend to slough off and float into the liquor folliculi and are readily noticeable. Such sloughing results in shrinkage of the cumulus oophorus and smoothing of its antral surfaces, a process that allows the ovum to eventually enter the liquor folliculi.[40] In the rat, the zona pellucida undergoes degeneration, followed rapidly by the oocyte. The granulosa cells also degenerate completely. However, the theca interna persists as interstitial masses, which eventually break up into small groups of cells scattered within the medulla of the ovary.

The growth and ultimate selection of follicles in the cycling rat ovary for preovulatory growth and maturation begin upon the transformation of the primordial follicle to a primary follicle, through a growth process that is independent of gonadotropin stimulation. Later stages of follicular growth are dependent upon gonadotropin support. Using a classification system for developing mouse follicles proposed by Pedersen and Peters,[43] Richards and Midgley[44] developed a model for follicular selection. For descriptive purposes, follicles are classified as (1) small preantral (primary) follicles just entering the pool of committed, growing follicles, which is distinguished from the large pool of quiescent, nongrowing primordial follicles; (2) large preantral (secondary) follicles; and (3) large antral (tertiary) preovulatory follicles. Some follicles begin to grow from the primordial stage each day of the cycle. This growth continues until either atresia or ovulation occurs. Those follicles that do ovulate on the morning of estrus actually began to grow 19 days earlier as a member of a larger group of growing primary follicles. These follicles continue to grow to large preantral stages. At this stage, the follicles either become atretic or, during the proestrus preceding the cycle in which they will ultimately rupture, are "committed" to the ovulatory pool. This model proposes that the follicles must arrive at the "pre-committed" stage in synchrony with the anticipated surge of FSH and LH from the pituitary gland. Those asynchronous follicles degenerate if gonadotrophin stimulation does not occur within a critical time.

By mapping the size distribution of large antral follicles (>390 μm diameter) on each day of the rat estrous cycle, one can observe a distinct variation on each day.[45,46] On late proestrus, preovulatory follicles are 500–750 μm in diameter just prior to rupture. On estrus, after ovulation and during metestrus, only follicles between 390 and 500 μm in diameter are present. During diestrus, follicles greater than 500 μm in diameter first appear. The absence of 390- to 500-μm diameter follicles on proestrus suggests that follicles of this size on late estrus and early metestrus are the ones that were selected to grow through diestrus and early proestrus and ultimately destined to ovulate.[45] Moreover, the absence of 390–500 μm diameter follicles on proestrus followed by their dramatic appearance on estrus further suggests that an event on proestrus selects these follicles to begin growth for the next estrous cycle. The hormonal control of this selection process will be described in another section.

Ovulation

The follicular events immediately preceding rupture and expulsion of the egg are easily visualized and were first described in the rabbit.[37,38] It is believed that the ovulatory event and its controls are generalizable to most mammals and are described in detail in Chapter 22. Rather than describe the changes from proestrus through ovulation, Blandau[29] treated immature rats with gonadotrophic preparations and observed the process of ovulation in over 300 follicles. The ovulatory follicles bulge from the ovarian surface and appear turgid and highly vascular. Within minutes after stimulation by gonadotrophic hormone, the macula pellucida or stigma forms at the apex of the preovulatory follicle. This is the first sign that the ovulatory process has begun. Beyond the periphery of the stigma, a dilated ring of blood vessels forms. As follicular rupture approaches, the superficial germinal epithelial cells covering the stigma break into small clumps. The underlying stroma thins and a delicate vesicle that may be a bleb of viscous follicular fluid forms. The actual escape of the ovum following breakdown of the stigma takes approximately 12 min. First, the granulosa cells begin to ooze through the plugged orifice, followed very quickly by the remaining egg mass. Once the egg is extruded, a great burst of follicular fluid follows. In the majority of cases, the cumulus and egg do not lie next to the rupturing stigma. Under these circumstances, the follicular fluid bursts through

as if under pressure. The cumulus and oocyte would be extruded subsequently in a stream. The oocyte rapidly finds its way to the ciliated fimbriated end of the oviduct. Within an hour after ovulation, eggs surrounded by cumulus masses can be recognized in the upper third of the oviduct. All ovulations are complete in 1.5 h.

Formation and Activation of the Corpus Luteum

During the estrous cycle of the rat, three or more generations of corpora lutea may be present on the ovary from the immediately preceding ovulatory cycles.[19] It appears that the cells of the corpus luteum have a dual origin. That is, based on anatomical and histochemical observations,[47] the corpus luteum arises from both the granulosa and thecal cells of the preovulatory follicle. Each set of corpora persist morphologically for 12–14 days. In the rat, each cyclic set of corpora lutea can be distinguished on the basis of size,[19,48] vascularity[49] and stain characteristics.[34,50,51] By diestrus, the newly formed corpora have attained their maximal size, which is maintained through metestrus of the following cycle. By diestrus of that second cycle, the corpora lutea abruptly regress. This regression coincides with closure of blood vessels, the appearance of areas of degeneration, leukocytic infiltration,[49] increase content of 20α-hydroxysteroid dehydrogenase,[50] and increase cholesterol content.[51] Such corpora lutea, if present in an unmated animal, are referred to as "nonfunctional" corpora lutea. That is, the corpora lutea do not secrete sufficient progesterone to support a decidual reaction (Chapter 23 and 24) induced by mechanical or chemical trauma of the uterine endometrium. Actually, the corpora are not completely nonfunctional because progesterone is secreted by the newly formed corpora lutea from metestrus through diestrus.[52] In the absence of luteotrophic support, however, they do secrete copious amounts of another progestin that is a metabolite of progesterone, 20α-hydroxyprogesterone. This steroid will not support a decidual reaction nor hold the hypothalamo–pituitary machinery controlling ovulation in check.

As the secretion of luteal progesterone declines through diestrus, a new ovulation takes place shortly thereafter. Thus, the "luteal phase" of rats (as well as hamsters and mice) is atypical. Other mammals have relatively long luteal phases (11–14 days) with extensive release of high levels of progesterone, while the corpora lutea of unmated rats secrete lower levels of progesterone for only 1–2 days. This serves as a basis for the short cycles in rodents. If, on the other hand, the animals are allowed to mate and become pregnant, the animals mate with infertile males, or the uterine cervix is stimulated artificially, the pituitary gland secretes sufficient quantities of a "luteotrophin" to "rescue" the corpus luteum and allow it to persist for a number of days, approximating a typical mammalian luteal phase.[52] If the mating is fertile, this luteal phase persists for the entire period of pregnancy, 20–22 days. If the mating is infertile or artificial, the corpus luteum persists for 12–14 days. This period is known as pseudopregnancy. The luteotrophin released by the mating stimulus is pituitary prolactin (PRL). The physiological basis for this extended luteal phase of the rat brought about by the mating stimulus will be described in a later section of this chapter.

In addition to support for luteal function provided by the pituitary gland, there is abundant literature to suggest that the placenta secretes a luteotrophin, which maintains the corpus luteum of the latter part of pregnancy. Moreover, compelling evidence leads us to believe that the decidual tissue may contain activity, independent of the well-established pituitary and varying placental activities,[53–57] that can serve as a luteotrophin.[58] Finally, although corpus luteum regression or luteolysis could be merely the consequence of withdrawal of luteotrophic support, there is ample evidence that the uterus may secrete "luteolysins," which directly or indirectly hasten luteal death[59] (Chapter 23).

Secretion of Ovarian Steroids during the Estrous Cycle

The preovulatory period of the estrous cycle is characterized by a growth of ovarian follicles and a concomitant enhanced secretion of estrogens, principally estradiol 17β. The secretion rate of estradiol into ovarian venous plasma is low on estrus, begins to rise significantly by late metestrus through the morning of diestrus and reaches peak concentrations by 12:00 h on the afternoon of proestrus.[59,60] The mean peripheral plasma concentration of estradiol (Figure 26.2) reflects the pattern found in ovarian vein blood.[52,61–64] That is, in the 4-day cycling rat, peripheral plasma levels of estradiol are basal through estrus. In late metestrus through early diestrus, plasma levels begin to rise. This increase continues through diestrus and early proestrus to reach peak values and plateau by mid-proestrus. During the early evening, shortly before the dark interval in the colony, estradiol levels fall rapidly, reaching basal values by the early morning hours of estrus. The cell types responsible for the secretion of estradiol throughout the cycle have been described in experiments by Falck.[65] Cotransplants of estradiol-sensitive tissue, the vagina, and various ovarian cellular compartments were made to the anterior chamber of the eye. Only transplantation of both theca interna and granulosa cells caused typical "estrogenic" changes in the cotransplanted vaginal tissue.

In rats with 4-day estrous cycles, peripheral plasma levels of testosterone and androstenedione are similar in pattern to estradiol.[66,67] The similar pattern of secretion of the androgens and estrogens reflect the fact that the synthesis, secretion, and controls of the two classes

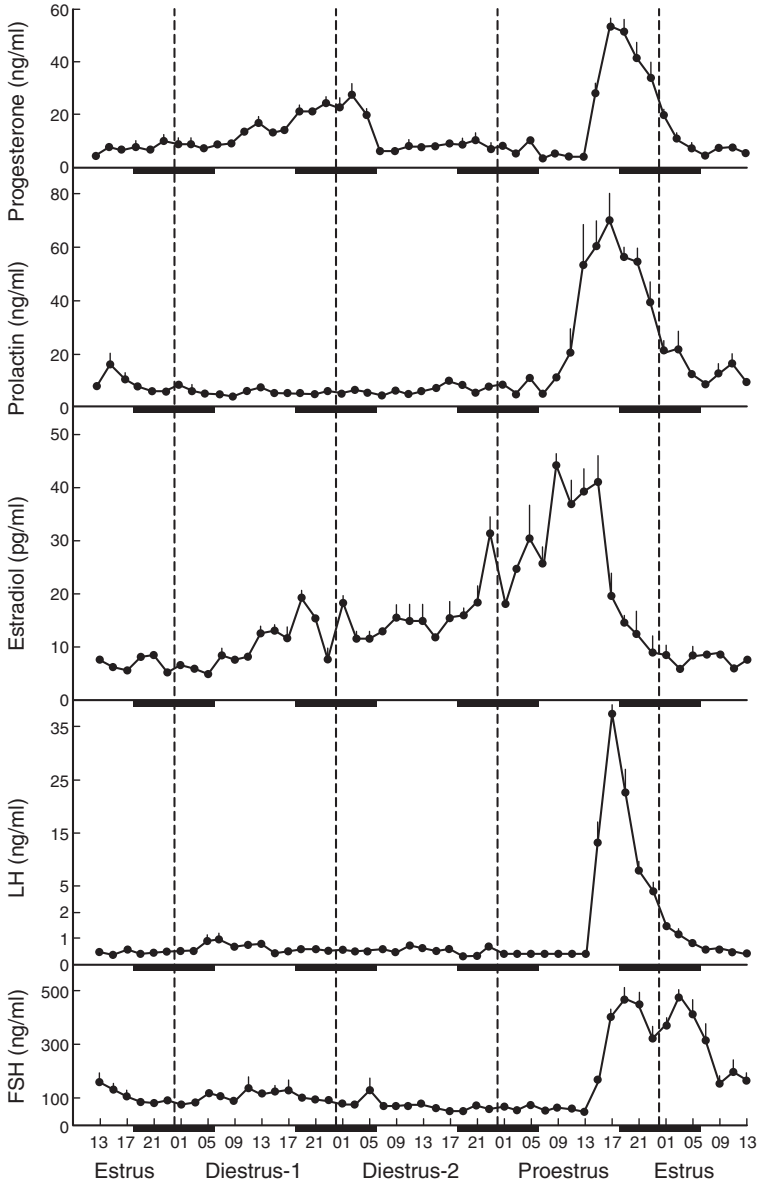

FIGURE 26.2 Concentrations of progesterone, prolactin, estradiol, LH, and FSH in peripheral plasma obtained at 2-h intervals throughout each day of the 4-day estrous cycle of the rat. Each point represents the mean hormone concentration (±SE) of five to six rats. Black bars represent the dark interval in the animal room (06:00–18:00) and the numbers below them represent the time-of-day (10–2) in terms of the 24-h clock. *From Ref. 52.*

of hormones are interrelated by the combined actions of FSH on granulosa cells and LH on theca interna cells, the so-called two-cell/two-gonadotropin model for estradiol biosynthesis (see Chapter 28).

In adult rats, the dominant progestin secreted into ovarian vein blood during the estrous cycle is 20α-hydroxypregn-4-en-3-one,[68–72] also known as 20α-hydroxyprogesterone (20α-OH-P). This progestin is a metabolite of progesterone, and its synthesis is catalyzed by the enzyme 20α-hydroxysteroid dehydrogenase.[73] 20α-hydroxyprogesterone is relatively inactive in classic progesterone bioassays. That is, it will not support the development of deciduomata in pseudopregnant rats.[74,75]

There are two peaks of both 20α-hydroxyprogesterone and progesterone secretion into ovarian vein blood during the cycle. The first peak occurs during the afternoon of metestrus and both steroids probably arise from the newly formed corpora lutea.[68,69] The second peak occurs during the late afternoon of proestrus.[68,69] The 20α-hydroxyprogesterone peak at this time probably comes from the corpora lutea, while the progesterone arises from the granulosa cells of the preovulatory follicle.[76] The secretion of these progestins into ovarian vein blood is mostly mirrored in their pattern of secretion in peripheral plasma.[52,62,64,77–79] In 4-day cycling rats, the secretion of 20α-hydroxyprogesterone into peripheral plasma appears to be quite variable. The lowest levels are observed during the morning of diestrus. However, peripheral levels rise during the evening of diestrus and the morning of proestrus to reach peak levels by 20:00 h on proestrus. The secretion of progesterone is more

regular (Figure 26.2). A large increase of follicular origin takes place during the afternoon and evening of proestrus. This increase occurs nearly simultaneously with the major ovulation-inducing increase of LH secretion. It reaches peak levels around the time of the LH peak in the early evening and returns to basal levels by the morning of estrus. A second major peak of luteal origin begins about midday on metestrus, is protracted through the early morning of diestrus, and falls to basal levels shortly after the onset of illumination on diestrus. This rise and fall represents the unsuccessful attempt of the newly formed corpus luteum to survive. The control of these patterns of progestin secretion during the estrous cycle as well as their physiological significance will be discussed in subsequent sections.

PATTERNS OF PITUITARY HORMONE SECRETION AND mRNA LEVELS DURING THE ESTROUS CYCLE

The predominant function of FSH is to stimulate estradiol secretion and growth of ovarian follicles, while LH induces ovulation and formation of corpora lutea and stimulates ovarian steroid hormone production. Both FSH and LH are each composed of two peptide subunits in virtually all mammals.[80] One subunit, designated α, is common to FSH, LH, and thyroid-stimulating hormone (TSH). The other subunit, β, is structurally specific for each gonadotropin. This specificity is responsible for the characteristic biologic activity of the intact (dimeric) molecules. Each gonadotropin has characteristic carbohydrate moieties, and these carbohydrate components may play a role in separate biologic activities.[80] The other hormone of the anterior pituitary gland of significance to the ovarian cycle of the rat is PRL, a single-chain peptide secreted from lactotropes (see also Chapter 12). Besides its well-known "pro-lactational" role shared by all mammals, pituitary PRL participates in maintenance of corpora lutea in rats, mice, and hamsters.

Control of the ovarian cycle of the rat by anterior pituitary hormones has been elucidated by various tools used to measure spontaneous release of the hormones during the estrous cycle. Prior to the advent of sensitive and specific radioimmunoassays, LH was measured by ovarian ascorbic acid depletion bioassay[81] and FSH by direct augmentation of the ovarian weight response to human chorionic gonadotropin (hCG)[82] or indirectly by uterine weight enhancement.[83] PRL activity was quantitated by the growth of the pigeon crop sac.[84] These early biological assays all had the distinct advantage of outstanding hormonal and tissue specificity, yet they shared the inherent disadvantage of lack of sensitivity such that cyclic patterns of pituitary hormone secretion into blood had to be inferred on the basis of changing content in the pituitary gland. Blood hormone levels were largely undetectable in samples obtained from individual animals. The concentration of hormone in the gland is the result of a balance between synthesis and release. Because these processes can vary independently of each other, declining levels of hormone in the gland may reflect decreased synthesis and release or release overtaking synthesis. Conversely, elevated levels may be the result of decreased release in the face of unchanging synthesis or acceleration of synthesis with or without compensatory increased release. For these reasons, the only definitive endpoint for hormone release is the concentration in blood. Sensitive radioimunoassays became available for rat FSH and LH[85] as well as rat PRL,[86] which permitted detection of basal serum levels of these hormones.

The development and availability of materials for these assays through the National Institutes of Health has represented a major milestone on our road to acquiring knowledge of the control of the ovarian cycle. The following descriptions of the pattern of pituitary hormone secretion are syntheses of the work of several laboratories[52,62,64,76,77] using these materials. All rats were housed under 14:10 or 12:12 light–dark cycles, with noon being the midpoint of the light period.[87,88] Under these conditions, the majority of the cycles are 4 days in length.[87] This description will be confined to animals with 4-day ovulatory cycles. The actual secretory patterns of LH, FSH, and PRL are shown in Figure 26.2 along with the circulating patterns of estradiol and progesterone. The interested reader is referred to the classic work of Nequin, Alvarez, and Schwartz[64] for a detailed description of differences in 4- and 5-day cycles.

Luteinizing Hormone

The serum levels of LH are at the lowest from early on the morning of estrus, shortly after ovulation, through metestrus, diestrus, and midday on proestrus (Figure 26.2). Although most studies report basal, unchanging levels of LH over this time,[52,62,64,76,77] one study reports slight but significant diurnal variation throughout the cycle.[89] This circadian pattern from estrus through diestrus consisted of a small elevation of LH each day at the midpoint of the light period and the lowest levels by midnight of each day. On the afternoon of proestrus, about 14:00 to 15:00 h, the circulating levels of LH begin to increase rapidly and ultimately reach peak levels by 17:00 to 19:00 h on that same evening (Figure 26.2). This rapid "surge" of LH induces follicular rupture and ovulation. Thereafter, these blood levels begin to decline and reach basal levels by early on the morning of estrus. When blood samples are obtained at 6- to 10-min-intervals during each day of the estrous cycle, a pulsatile or ultradian

pattern of LH secretion is observed.[90–93] Specifically, the pulses occur at periodicities of around 55–60 min and amplitudes, expressed in terms of the original NIDDK-LH-RP-1 standard, of 15–40 ng/ml whole blood from metestrus through the morning of proestrus (Figure 26.3).

During the afternoon of proestrus, the ovulation-inducing surge of LH has been characterized as composed of either declining pulse frequency with an amplitude of 600 ng/ml[92] or a single constant almost unvarying linear rise of LH secretion with an amplitude of nearly 200 times baseline in some animals (Figure 26.4).[89,90,94] This single surge declines through the early morning of estrus. By the late morning and throughout the day of estrus, pulses of LH are infrequent[91] or absent.[90] The circhoral pulses return by the morning of metestrus and continue through proestrus as before. The control of this unique pattern of LH secretion will be described in later sections.

Follicle Stimulating Hormone

The pattern of FSH secretion throughout the majority of the estrous cycle is similar to that of LH.[52,62,85,89] Specifically, basal levels of FSH are secreted from late estrus through metestrus, diestrus, and midday on proestrus (Figure 26.2). From mid-afternoon onward, proestrus FSH secretion increases simultaneous with that of LH. That is, by 14:00 to 15:00 h, the circulating levels of FSH begin to increase rapidly and ultimately reach peak levels by 17:00 to 19:00 h that same evening. Although both FSH and LH levels begin to decline to baseline after this time, during the early morning of estrus a secondary rise of FSH begins and peaks shortly thereafter. Circulating levels of FSH then begin to decline and reach baseline by the early evening of estrus. This early initial phase of FSH secretion shares some controls with the contemporaneous LH "surge" while the estrous phase of FSH secretion is under different controls, as described later.

Circadian and ultradian pulses of FSH similar to LH have not been described during the estrous cycle.[52,62,85,89] That is not to indicate that they do not exist, but merely that the available FSH radioimmunoassays are not sensitive enough to distinguish fluctuations in low levels of FSH secretion during the estrous cycle. On the other hand, pulsatile LH and FSH secretion does occur in the gonadectomized animal, but the interpulse interval of LH is significantly shorter than that of FSH.[95] The similarities and differences in control of FSH and LH secretion during the estrous cycle will be described in a later section.

Prolactin

The pattern of PRL secretion throughout the estrous cycle is also similar to that of LH[52,62,85,89] (see also Chapter 12). That is, basal levels of PRL are secreted from the evening of estrus through the early morning of proestrus (Figure 26.2). During the afternoon of proestrus, a "surge" of PRL, similar in timing to that of LH, is observed.[52,89] Although most laboratories have

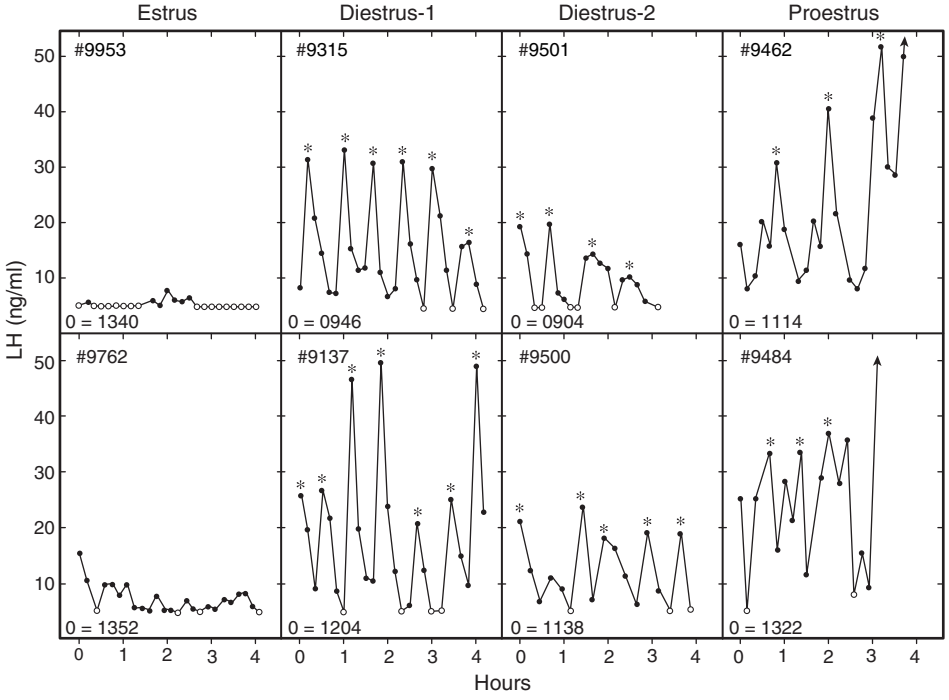

FIGURE 26.3 Concentrations of LH in peripheral plasma obtained at 10-min intervals for 4 h from each of two representative animals during each day of the 4-day estrous cycle of the rat. The animal numbers are presented in the upper left corner of each block and the time of sampling initiation is given as zero-time in the lower left. Statistically defined pulses are indicated with asterisks and the open circles represent values below the detection limits of the assay. *From Ref. 89.*

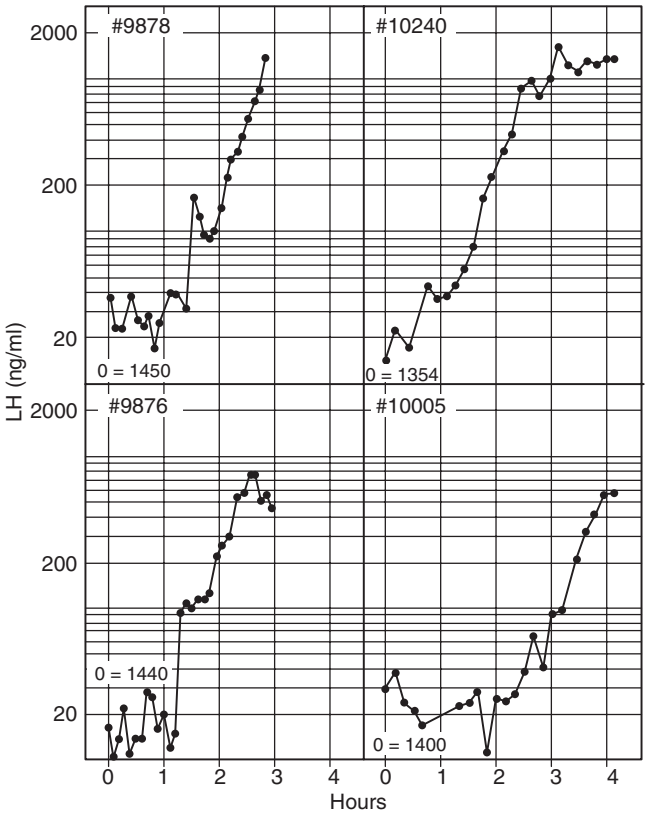

FIGURE 26.4 A semilog plot of the LH concentration in blood samples obtained at 6-min (left panels) or 10-min (right panels) intervals during the afternoon of proestrus. The details are described in Figure 26.3. Note the absence of discrete multiple pulses of LH during the preovulatory surge during proestrus. *From Ref. 89.*

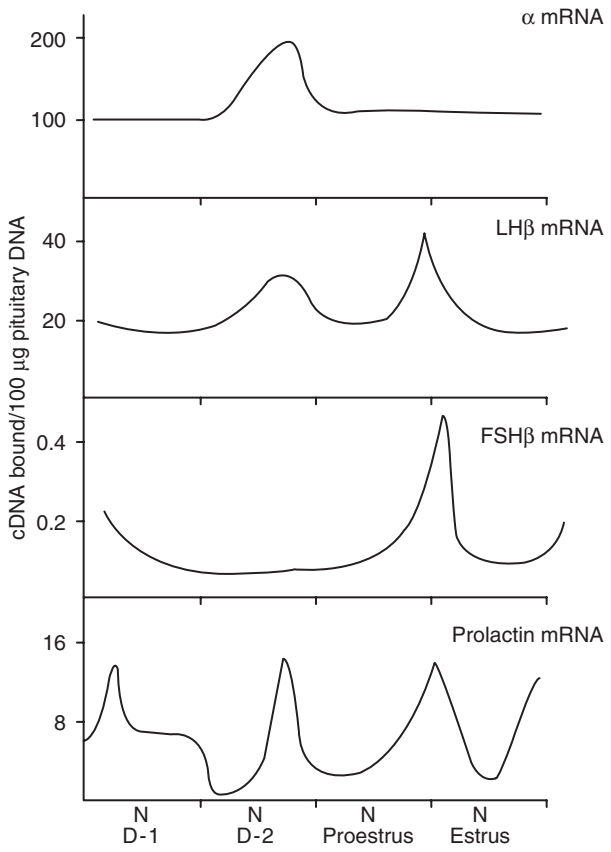

FIGURE 26.5 Pituitary content of mRNA, LH-β mRNA, FSH-β mRNA, and prolactin mRNA throughout the estrous cycle of the rat. *Redrawn from Refs 97–99,101.*

described a single surge of PRL on proestrus,[52,89,96] other laboratories have reported a secondary increase on estrus[62] or continuously elevated PRL levels on proestrus, estrus, and metestrus.[97] Because these latter patterns may have been due to method and frequency of blood collection, it is generally accepted that the afternoon of proestrus is the only time that a major "surge" of PRL secretion occurs.

Pituitary Gonadotropin Subunit and Prolactin mRNAs

Gene expression for the α subunit,[98] the β subunit of LH,[98] the FSH-β subunit[98] and PRL[99,100] have been described in the anterior pituitary gland throughout the estrous cycle of the rat (Figure 26.5). Alpha mRNA is heightened by 08:00 h diestrus-2 reaching maximal values by 20:00 h and declining abruptly by 24:00 h.[101] The content of α-mRNA remains low during proestrus, estrus, and diestrus-1. In contrast, LH-β mRNA is low during estrus and diestrus-1, increases at 08:00 h during diestrus-2, and then falls rapidly and remains low throughout the morning of proestrus.[101] By 14:00 h on proestrus, a second increase begins, which peaks at 17:00 h and then declines rapidly. This peak precedes the preovulatory surge of LH secretion. By 22:00 h proestrus, the levels are equivalent to estrus and diestrus-1. FSH-β mRNA increases from 20:00 h on proestrus to a peak value by 02:00 h estrus, returns to basal levels by 08:00 h, and remains low through 20:00 h estrus.[98] This peak corresponds to the surge of FSH secretion on proestrus. Another smaller increase begins by 23:00 h estrus, which remains elevated throughout the afternoon of diestrus-1, although FSH secretion remains basal over that time. The pattern of PRL mRNA during the estrous cycle is distinctly different than that of the gonadotrophin subunit messages.[99,100]

There is a striking increase of PRL mRNA content in the anterior pituitary gland at specific intervals during each day of the estrous cycle.[99,100] During proestrus and estrus, PRL mRNA is higher at 23:00 h than during any other time on each day. In contrast, PRL mRNA levels are highest at 08:00 h on diestrus-1. During diestrus-2, PRL mRNA values peak between 17:00 and 20:00 h. The only clear correlation between pituitary or plasma PRL and PRL mRNA is found on the afternoon of proestrus, when a morning rise in PRL mRNA precedes a morning increase in pituitary PRL content.[99]

NEURAL EVENTS DURING THE ESTROUS CYCLE

Circadian Control Mechanisms

As described above, frequent blood sampling reveals that LH secretion during the estrous cycle is largely pulsatile, with frequencies of less than one pulse per hour.[90–93] The pulses originate as the result of pulsatile release of GnRH presumably into the portal vasculature.[102] This is not the only patterned rhythmic control of the rat's ovarian cycle. Indeed, Everett and Sawyer[103] showed that the LH-release mechanism is under a 24-h photoperiodic control and thus can fall under the category of a circadian rhythm. These elegant classic studies (Figure 26.6) showed that administration of pentobarbital between 14:00 and 15:45 h on the afternoon of proestrus to animals under daily illumination from 05:00 to 19:00 h resulted in blockade of ovulation on estrus for 1 day (Figure 26.6(C)). Treatment with the same drug again the next day, estrus, blocked ovulation a further 24 h (Figure 26.6(E)). Treatment at times other than 14:00 to 15:45 h (Figure 26.6(B,D)) was much less effective in blocking ovulation on each day. These data led to the concept of a daily "critical period"—the interval during which a daily neural event leads to release of an LH surge and subsequent ovulation. The timing of the critical period can be shifted by altering the "zeitgeber" or time cue. That is, shifting of the phase but not the duration of the lighting period will shift the critical period by a corresponding time.[88] Thus, environmental light–dark cycles act to regulate ovarian cycles via entrainment of their circadian system. In fact, with prolonged exposure to the lack of a daily photoperiod, such as constant light, the rat will gradually become acyclic as manifested by constant vaginal cornification.[104]

A number of studies have implicated the paired suprachiasmatic nuclei (SCN) of the hypothalamus as the generators of the signal that is entrained to the light–dark rhythm. Neurons within the SCN function as a master circadian clock, consisting of a unified network of cells in which interlocked transcription-translation feedback loops generate 24-h rhythms. Retinohypothalamic pathways communicate photic signals that entrain these rhythms[105–107] and SCN efferents convey output signals that appropriately time the initiation of GnRH (and therefore LH) surges to occur during the critical period on the afternoon of proestrus. Lesioning or isolation of the SCN terminates the estrous cycle in rats,[108,109] presumably by preventing transduction of the signals for initiation of the surge. Similarly, mutation of the circadian locomotor output cycles kaput (*Clock*) gene, a core component of the molecular circadian pacemaker, renders female mice incapable of releasing LH surges at the expected time on the afternoon of proestrus (Figure 26.7).[110] Thus, one of the signals triggering release of the

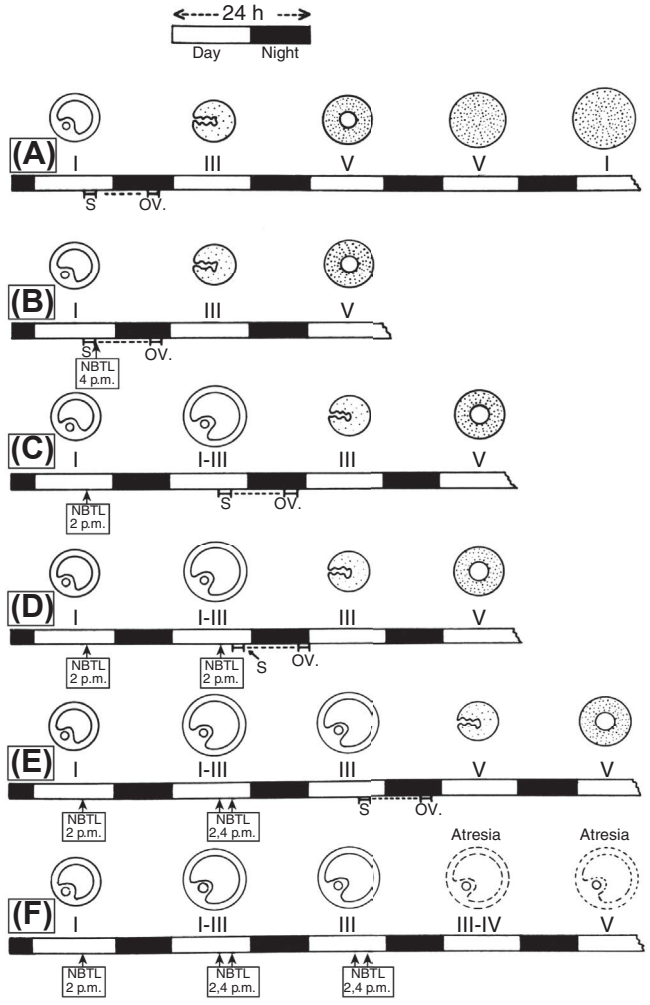

FIGURE 26.6 The classic experiments of Everett and Sawyer[103] illustrating the experimental basis for the 24-h periodicity in the LH-release mechanism during the estrous cycle of the rat. The light and dark bars represent a 24-h continuum; s defines the critical period and ov the time of ovulation. The Roman numerals represent vaginal smear stages: in each group (A–F) I represents the afternoon of proestrus. Group A represents the timing of the events of the normal cycle from ovulation through corpus luteum formation. Administration of the central anesthetic Nembutal (NBTL, sodium pentobarbital) at 4 p.m. on proestrus does not disturb the timing of the normal cyclic events (Group B). However, a single injection of NBTL at 2 p.m. on proestrus postpones the release of a surge quantity of LH and ovulation 24 h (Group C). Repetition of the treatment at the same time the following day, estrus, was insufficient to block LH release and ovulation and ovulation an additional 24 h (Group D). On the other hand, administration of NBTL at 2 p.m. on proestrus and at both 2 and 4 p.m. on estrus blocks the surge of LH and ovulation 48 h (Group E). The follicles cannot remain competent to respond to a delayed LH surge beyond 72 h. Consequently, atresia results from such a delay (Group F). These experiments have indicated that a hypothalamic signal occurs daily between 2 and 4 p.m., the critical period, which leads to LH release. However, the ovarian steroid milieu is appropriate for such release only on proestrus.[103]

LH surge and subsequent ovulation is provided by the circadian clock so that there is only a limited "window" during each day when the LH surge could potentially be initiated. This daily potential for LH release is determined by transduction of the lighting periodicity, the

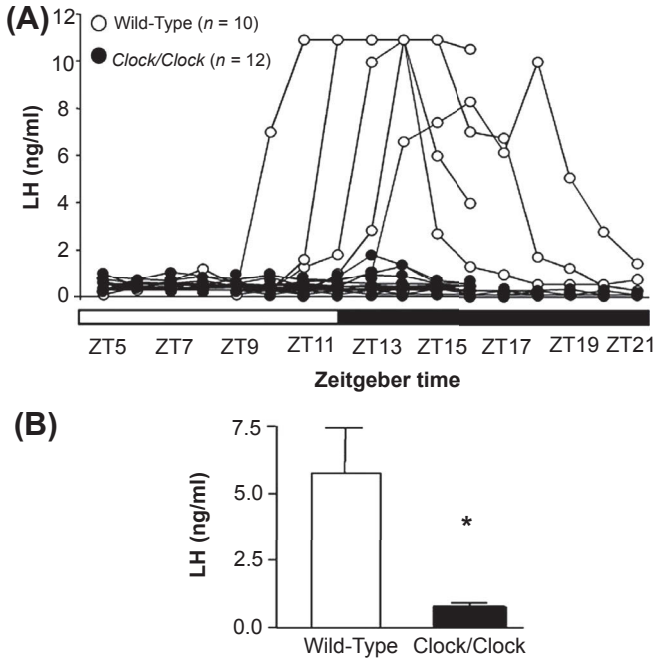

FIGURE 26.7 ***Clock/Clock*** **mutants fail to have a coordinated LH surge on the day of proestrus.** (A) Individual LH traces from all wild-type (open circles) and *Clock/Clock* (closed circles) females sampled from either 0500–1600 or 0900–2100. On the *x*-axis, white bar denotes lights on and black bar denotes lights off. (B) Peak LH values observed in wild-type versus *Clock/Clock* female mice $^{*}P<0.01$. *Figure adapted from Ref. 110.*

zeitgeiber, by the SCN. It is likely that the specification of the afternoon hours for release of LH surges serves the adaptive purpose of bringing into temporal register behavioral heat, which commences on the evening of proestrous, with ovulation in the early hours of estus. The interaction of ovarian hormones with circadian clock signals eventuating in LH, FSH, and PRL release will be described in the next sections.

Control by GnRH

GnRH is synthesized by neurosecretory neurons in the basal forebrain, transported along axons to neurovascular junctions in the ME, and released in a pulsatile fashion into the hypothalamic–hypophysial portal vasculature (Figure 26.8). The cellular substrates of pulsatile GnRH release have been referred to as the hypothalamic GnRH pulse generator.[111] Details of the molecular mechanisms that govern GnRH gene expression, as well as the electrophysiological and cellular events that may govern GnRH pulsatility, are discussed in depth in Chapters 11 and 27.

The pulsatile mode of GnRH release has been clearly shown to be required for the continued stimulation of "basal" LH and FSH secretions,[112] although in rats FSH secretion appears to have a GnRH-independent, and perhaps constitutive, component.[113] Basal secretions of the

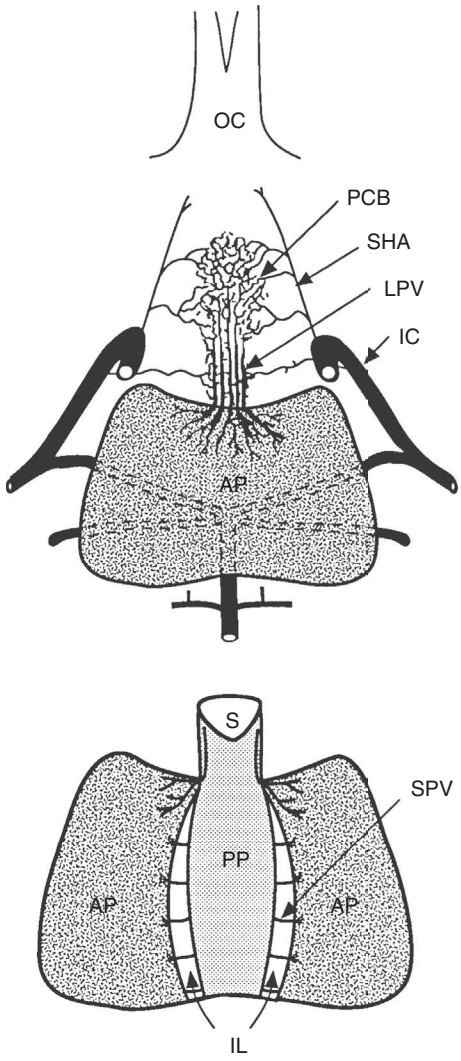

FIGURE 26.8 **Diagrammatic representation of the blood supply to the anterior pituitary (AP) gland through the long and short portal vessels.** Top is a ventral view and bottom is a dorsal view. The superior hypophysial arteries (SHA) arise from the internal carotid artery (IC). The SHA, in turn, supplies blood to the primary capillary bed (PCB) in the median eminence (ME). The long portal vessels (LPV) arise from the PCB and pass down the stalk (S) to the AP. The short portal vessels (SPV) arise from capillaries in the posterior pituitary (PP), pass through the intermediate lobe (IL) to the AP. Thus neurohormones can arrive at sites of action in the AP from axons which terminate in the ME or the PP. OC, optic chiasm.

gonadotropins continue throughout estrus, metestrus, diestrus, and the morning of proestrus in the female rat. On the afternoon of proestrus, this pulsatile release mode is interrupted by the neurosecretion of a surge of GnRH, which triggers the preovulatory gonadotropin surge. The regulation of these two modes of GnRH release—basal pulsatility and surge secretion—are essential to the normal progression by the female rat through the stages of the estrous cycle. An absence or excess of GnRH pulsatility disrupts follicular maturation and steroidogenesis, and failure to release a preovulatory gonadotropin surge

results in anovulation. Much of the subsequent discussion of GnRH release will focus on the mechanisms by which ovarian hormones exert homeostatic negative feedback regulation of basal GnRH pulsatility, and the positive feedback mechanisms that permit release of preovulatory GnRH surges.

GnRH has been localized in the rodent brain using a number of imaginative approaches. Wheaton and coworkers[114] measured GnRH by radioimmunoassay in frozen sections of rat brain and found the majority of the immunoreactivity in the infundibular stem and ME, the site at which axon terminals and the primary capillary bed of the hypophysial portal system meet (Figure 26.9). In addition, smaller but significant quantities of GnRH were measured in the anterior hypothalamus, the SCN, and rostrally in the preoptic area. Similar observations were made by Palkovits and collaborators[115–117] and Selmanoff and collaborators[118] using a technique of punching tissue from specific brain regions. In addition, GnRH is found in circumventricular organs particularly the organum vasculosum of the lamina terminalis (OVLT), the vascular structure at the tip of the third ventricle within the preoptic area.

Using various immunocytochemical approaches, GnRH neurons have been shown to be small, typically fusiform, and distributed rostro-caudally from the diagonal band of Broca (DBB) to the retrochiasmatic region and extend up to 2 mm on either side of the midline in the preoptic region and medial forebrain bundle[119–126] (see Chapter 11). Mapping of GnRH mRNA-expressing soma by in situ hybridization (ISH) has revealed the same GnRH neuronal distribution pattern.[127–130] GnRH-producing cells are concentrated in the DBB, stria terminalis, medial nucleus of the septum, medial preoptic area (mPOA), and the anterior hypothalamic area (AHA). GnRH-containing perikarya have also been found in the retrochiasmatic area of the medial basal hypothalamus.[125,126,131] GnRH neurons that project into the ME are concentrated basally in the midline regions from the mPOA rostrally and through the retrochiasmatic area caudally. These fibers terminate on capillaries of the primary plexus of the hypothalamo-hypophysial portal vasculature.[132]

There is reasonably good correlation between the location of GnRH cell bodies and axons and experimental manipulations at these sites, which affect the ovulatory surge of LH. Using an experimental model in which the proestrous preovulatory surge of LH has been blocked, Everett[133] mapped a funnel-shaped septal-preoptico-tuberal pathway that responds to electrochemical stimulation by releasing an ovulatory amount of LH. These fibers are fan-shaped in the septal complex, narrow through the mPOA-AHA, and converge as a compact band in the ME (Figure 26.10). Electrochemical stimulation along this band excites LH release.[134] However, despite the presence of GnRH fibers, stimulation of the lateral preoptic area, the median forebrain bundle, and dorsal or ventral medial hypothalamus fails to excite LH release.[133,134] These studies demonstrate that a core band of GnRH fibers originating from septal and preoptic GnRH neurons mediate neurosecretion of the decapeptide at neurovascular junctions in the ME, while the additional contribution of fibers coursing more laterally remain unconfirmed. Early lesion studies suggested that the mPOA may function as an integrative center for the generation of LH surges[135,136]—a contention later refuted by findings that dysregulated PRL secretion in lesioned animals may have been responsible for the absence of cyclicity in these animals.[136,137] However, the lesion of discrete midline sites through this area such as the OVLT,[138] the medial preoptic nucleus (mPN)[139] or more specifically the anteroventral periventricular nucleus (AVPv), or SCN[135] produced anovulation and anestrus or constant estrus.

As described in a subsequent section, it is now well accepted that the release of GnRH surges and ovulatory cyclicity depend upon the integrity of both the SCN and the AVPv. SCN delivers the daily neuronal signal for release of the surge, whereas AVPv receives these neural signals and integrates them with the positive feedback actions of ovarian hormones that permit the initiation of the GnRH surge. The circuit is then believed to be completed by projections from the AVPv to GnRH neurons, which prompt release of a preovulatory GnRH surge into the hypophysial portal vasculature.[140,141]

Neurosecretion of the GnRH Surge

The integration of circadian and endocrine signals on the afternoon of proestrus triggers a cascade of events within the hypothalamus, eventuating in a 2–4 h increase in GnRH release from neurovascular terminals within the ME. This, in turn, is the stimulus for the ovulation-inducing surge of LH. Just as the secretion of the LH surge on proestrus is reflected in altered pituitary levels of LH,[104] so too is a surge of GnRH release into portal blood on proestrus reflected by changing levels of the decapeptide in the hypothalamus. Using indirect estimates of bioactive GnRH activity in hypothalamic extract by assaying their LH-releasing capabilities from pituitaries in vitro, there appeared to be enhanced LH-releasing activity in hypothalami around the time of the LH surge on proestrus and a rapid decline in hypothalamic content associated with the surge.[142–144] GnRH concentrations have also been measured in the brain by immunohistochemistry or radioimmunoassay. In confirmation of the earlier studies, it seems that GnRH activity of the medial basal hypothalamus (MBH) declines as the proestrous surge of LH begins[145,146] or as peak peripheral levels of LH are approached.[147–149] There

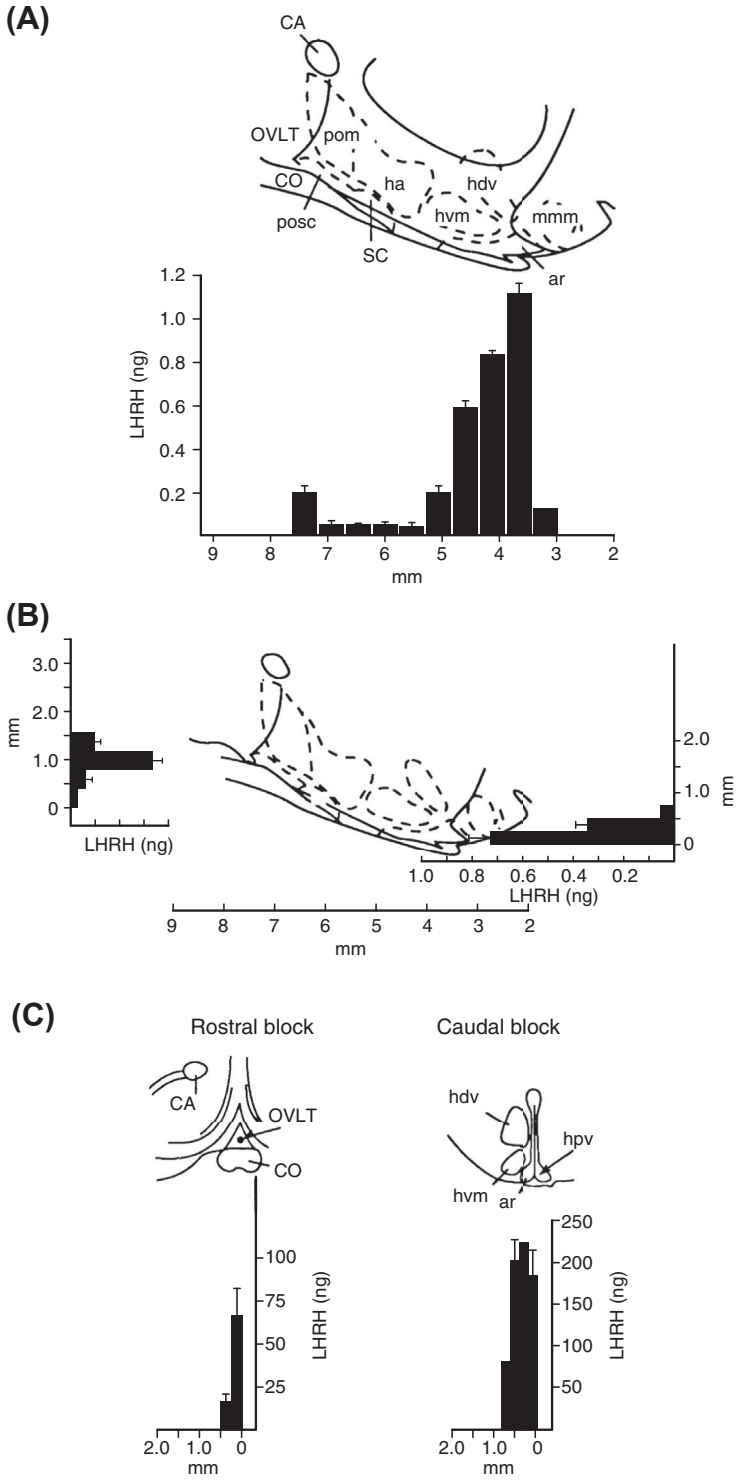

FIGURE 26.9 Immunoassayable LHRH (aka GnRH) in extracts of frontal (panel A), horizontal (panel B), and sagittal (panel C) sections of the rat brain. The bars of each histogram represent the mean (+SE) GnRH content in sections projecting through parasagittal (panels A and B) or frontal (panel C) images of the hypothalamus of the rat. ar, arcuate nucleus; ha, anterior hypothalamic nucleus; hdv, dorsomedial hypothalamic nucleus; hpv, periventricular hypothalamic nucleus; hvm, ventromedial hypothalamic nucleus; pom, medial preoptic nucleus; posc, pars suprachiasmatic-preoptic nucleus; mmm, pars medialis-medial mamillary nucleus; sc, suprachiasmatic nucleus; CA, anterior commissure; CO, optic chiasm; I, infundibulum; OVLT, organum vasculosum of the lamina terminalis. Note that the greatest content of LHRH (GnRH) was in a broad band corresponding to the ar with lesser but significant amounts in the OVLT. *From Ref. 17.*

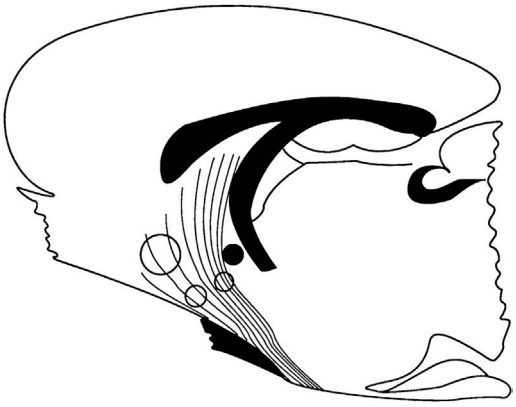

FIGURE 26.10 **Sagittal view of the rat brain diagramming the course of fibers which, when lesioned, affect ovulation.** Widely dispersed origins are in the septal complex where relatively large irritative lesions (large open circle) are required to activate the number of fibers that can be affected by small lesions (small open circles) in the preoptic area. *From Ref. 132.*

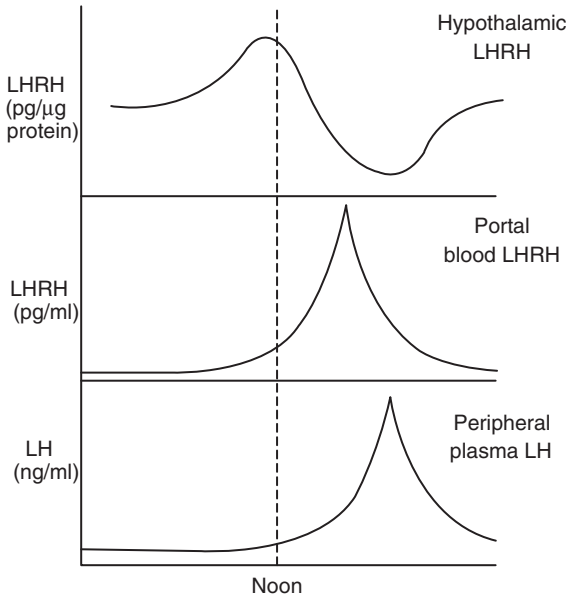

FIGURE 26.11 **Diagrammatic representation of the changes in hypothalamic concentration of LHRH (aka GnRH),[152] portal blood concentration of LHRH (GnRH),[154] and peripheral plasma concentration of LH[64] throughout proestrus in the rat.** Note that preceding the release of the ovulation inducing surge of LH there is a dramatic decline in hypothalamic concentration of GnRH and a corresponding increase in portal blood levels of GnRH.

does not appear to be general agreement on the increase in hypothalamic GnRH-content prior to the surge.[150–152] This may be due merely to variation in dissection technique of the various hypothalamic areas. In one study,[153] six areas along the preoptic-suprachiasmatic-tuberoinfundibular tract were dissected by the Palkovits' micropunch technique prior to and through the ovulation-inducing surge of LH on proestrus. Between 09:00 and 12:00 h on proestrus, there was a large increase in the concentration of GnRH in the ME as well as the SCN, mPN and anterior hypothalamic nucleus. Smaller, but significant, increases were observed in the retrochiasmatic area as well as the SCN. As with other studies, a large dramatic fall in GnRH concentration occurred in all areas between 12:00 and 15:00 h when the surge of LH begins. The pattern of GnRH mRNA expression levels as measured by ISH roughly mirrors these increases and then decreases in GnRH peptide concentrations in the mPOA and DBB.[130] Presumably, these changes are a reflection of GnRH gene expression and then release of GnRH into portal blood (Figure 26.11). Indeed, using a sophisticated surgical approach coupled with an anesthetic that does not appear to block the ovulatory surge of LH secretion on proestrus, Sarkar and collaborators[154] and later Ching[155,156] described an increase in radioimmunoassayable GnRH concentration in hypophysial blood, which shortly precedes the preovulatory surge of LH release on proestrus. Unfortunately, current methodologies as applied to rodents are such that it is difficult to obtain a reasonable amount of portal plasma frequently enough to determine if LH release on proestrus in the rat[90,92] is correlated with changes in GnRH pulse frequency and amplitude or merely the consequence of steroid-induced alteration in pituitary sensitivity to the decapeptide without a variation in GnRH frequency or amplitude.

In a larger mammal, the sheep, GnRH has been described as pulsatile in portal blood with a transition to a continuous mode of GnRH secretion during surge release (also see Chapter 27).[157] On the other hand, in the rat, by frequently perfusing the MBH with artificial cerebrospinal fluid and then sampling that fluid for assay of GnRH content (push–pull perfusion), Levine and Ramirez[102] described the patterns of release from GnRH terminals during the estrous cycle. Presumably, these are the neurons from which GnRH is secreted into hypophysial portal blood. Under these conditions, pulsatile GnRH release can be evaluated on proestrus. During proestrus, the average GnRH interpulse interval was 48 min. Pulses of increased amplitude and duration occurred throughout the afternoon hours, constituting a significant increase in the total GnRH release, with peak amount of GnRH release occurring between 1600 and 1800 h. It is this episode of release that presumably stimulates the release of the LH surge.[102] Using a microdialysis technique to monitor GnRH release, hormonally-induced GnRH surges have also been found to be comprised of pulses of increased amplitude in female rats[158] (Figure 26.12). These data, coupled with the observation that immunoneutralization of GnRH on proestrus blocks the surge of LH,[159] firmly establish the decapeptide as the physiological gonadotropin-releasing hormone that evokes the mid-cycle gonadotropin surge. The multiple pulses of GnRH likely serve a physiological role in the economy of LH release. Indeed, it has been shown that

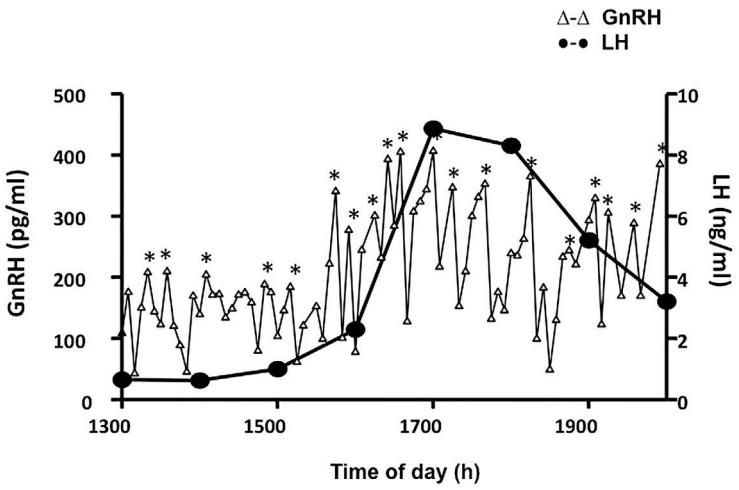

FIGURE 26.12 GnRH and LH surges in an ovariectomized, estradiol (E_2)-primed rat. GnRH release was measured by radioimmunoassay (RIA) in 5-min microdialysates of the mediobasal hypothalamus, and LH was measured by RIA in serum obtained hourly via an atrial catheter. The E_2 priming (30 μg E_2benzoate on the previous day) stimulated significant increases in GnRH pulse amplitude and mean level commencing at approximately 1600 h, accompanied by a concomitant 5-fold increase in LH. Asterisks represent significant pulses of GnRH release as determined by the ULTRA pulse analysis program. *Figure adapted from Ref. 158.*

small pulses of GnRH "prime" the gonadotroph cell to be maximally responsive to a subsequent release of a larger GnRH pulse on proestrus.[160,161]

Although the decapeptide has been unequivocally identified as the LH-releasing hormone in mammals, it is not clear that the termination of the LH surge is due exclusively to termination of GnRH secretion into hypophysial portal blood. Indeed, in sheep the GnRH surge significantly outlasts the duration of the LH surge.[157] It is possible that the termination of the LH surge reflects a limited capacity of gonadotropes to respond to heightened GnRH release. Alternatively, continued secretion of the LH surge may be actively suppressed by the pituitary actions of peptides of hypothalamic origins with gonadotropin inhibiting activity. One of these, the RF-amide orthologue of the avian gonadotropin inhibiting hormone (GnIH),[162] is considered more fully as a direct modulator of GnRH neurons later in this chapter. A second peptide of hypothalamic origin that inhibits GnRH-stimulated LH secretion has been partially characterized,[163] and administration of antisera to the peptide was shown to result in heightened release of LH on proestrus or in response to a challenge with estradiol.[164]

Other Neurohormones Controlling LH and FSH Secretion

Although GnRH occupies the primacy as the gonadotropin-releasing hormone, other neurosecretory products have been suggested to play complementary roles by regulating the responsiveness of gonadotropes to the stimulatory actions of GnRH. The list of putative "GnRH responsiveness factors" is headed by oxytocin (OT), neuropeptide Y (NPY) and galanin, each of which has been shown to have the capacity to regulate pituitary responsiveness to GnRH during the generation of preovulatory LH surges. There is evidence that OT acts at the anterior pituitary gland to augment the stimulatory effects of GnRH on LH secretion.[134,165–176] OT is found in portal blood[177,178] in highest amounts on the afternoon of proestrus.[178] Intravenous administration of an inhibitor to OT on the afternoon of proestrus blocks the preovulatory release of LH.[179] Passive immunization of OT, however, does not reduce the magnitude of the LH surge,[180] and OT- null mutant mice exhibit normal fertility.[181] Thus, the role of OT as a GnRH-responsiveness factor appears to be a supportive one, but not in itself obligatory for normal ovarian cyclicity. There is also abundant evidence that NPY acts a neurohormone to modulate GnRH-stimulated LH secretion.[182,183] NPY is found in the hypophysial portal circulation in high concentrations.[184,185] It regulates the binding of GnRH to its receptor on the gonadotroph cell membrane,[186,187] potentiates GnRH-stimulated LH surges,[188] and modulates calcium-transients in gonadotrophs.[189] These effects are probably mediated through the Y-1 receptor.[190] Hypothalamic NPY gene expression increases prior to the onset of the LH surge.[191,192] This suggests a role for NPY as a neurohormone participating with GnRH to regulate LH secretion at the gonadotroph. This idea has obtained direct support from the finding that genetic ablation of NPY in female mice results in a significant attenuation of both spontaneous and steroid-induced LH surges.[193] Finally, there is evidence for a neuromodulatory, neuroendocrine, and paracrine/autocrine role for galanin in the control of pituitary LH secretion. Galanin can stimulate LH secretion[194] and enhance GnRH-stimulated LH secretion in vivo.[195] It may act as a neurohormone since it is colocalized with GnRH[196] and has been found in portal blood.[197] Whether these actions of galanin are significant for the control of LH secretion is unknown at this time.

Though GnRH will release both FSH and LH,[198] there is still some uncertainty if one or possibly two releasing hormones exist. In the latter case, GnRH could release both LH and FSH while a distinct FSH-releasing factor

(FSH-RF) may exist. Early attempts to identify a separate FSH-RF provided some circumstantial evidence for such a molecule; however, no such factor has been isolated and identified. Radiofrequency lesions placed in the paraventricular nucleus-dorsal AHA (PVN-dAHA) were found to lower the plasma FSH levels of ovariectomized rats without disturbing the blood levels of LH or the concentration of GnRH in the ME.[199] Similarly, electrochemical stimulation of the DAHA caused FSH but no LH release.[200] Moreover, the posterior ME has greater FSH-releasing activity than can be accounted for by its content of GnRH.[201] Finally, a bioactive FSH-RF was isolated by size exclusion and ion exchange chromatography.[202,203] Fractions with greater FSH-releasing activity than could be accounted for by immunoreactive GnRH alone were detected prior to the immunoreactive GnRH peak. If indeed a distinct FSH-RF does exist, it may play an important role in the biphasic pattern of FSH secretion on proestrus through estrus. Administration of antiserum to GnRH[204,205] or a GnRH antagonist[206] blocks the preovulatory surge of LH, but the secondary surge of FSH on estrus is undisturbed by these treatments. These data, taken together, suggest one scenario in which a distinct FSH-RF may control the secondary surge of FSH on estrus. However, other studies (described later) argue that the secondary surge is largely independent of hypothalamic control.

The physiological control by peptidergic neurohormones of the secretion of PRL during the estrous cycle is less clear than that of LH and FSH. A number of neuropeptides have been demonstrated to stimulate PRL release. However, the most likely candidates as neurohumoral PRL-releasing hormones (PRHs) during the estrous cycle are thyrotropin-releasing hormone (TRH), vasoactive intestinal polypeptide (VIP), and OT. All three peptides are found in hypophysial portal blood[177,178,207,208] and stimulate PRL-release in vitro[209,210] and in vivo.[209] The indication that TRH plays a physiological role in the release of PRL during the estrous cycle comes from the observation that antiserum to TRH attenuates the basal secretion of PRL on diestrus and the surge on proestrus.[211] In TRH gene knockout mice, however, the absence of the peptide was not found to be associated with alterations in serum PRL levels or PRL content of the pituitary gland during proestrus.[212] The notion that VIP may be involved in the regulation of PRL secretion during the estrous cycle is supported by the fact that an antiserum to VIP administered on the afternoon of proestrus attenuates the peak phase of the PRL surge later that day.[213] However, the levels of VIP in the hypothalamus and anterior pituitary do not change during the surge of PRL on proestrus.[214] Therefore, a role for VIP in the cyclic release of PRL remains uncertain. The concentration of OT in hypophysial portal blood increases on the afternoon of proestrus just prior to the surge of PRL release into peripheral plasma.[178] Peripheral administration of an OT antagonist on the afternoon of proestrus completely blocked the proestrous surge of PRL.[179] Such data provide compelling evidence for a role of OT in the release of PRL on the afternoon of proestrus.

A member of the RF-amide family of peptides was originally shown to stimulate PRL secretion from cultured anterior pituitary cells and was therefore named PRL-releasing peptide (PrRP). Although PrRP has been implicated in a variety of other physiological functions, its importance in the physiological regulation of PRL in rodents has been questioned, especially in light of the finding that it is not detectable in the ME and has inconsistent PRL-releasing properties in vivo[215,216] (see also Chapter 12).

CONTROL OF PITUITARY HORMONE SECRETION

The cyclic release of LH, FSH, and PRL during the estrous cycle (Figure 26.5) is the result of positive (stimulatory) and negative (inhibitory) feedback control by the ovarian steroids acting at both the hypothalamus and pituitary gland. The positive feedback actions of ovarian steroids culminate in the release of the mid-cycle GnRH and LH surges that trigger ovulation. The negative feedback actions of ovarian steroids and additional ovarian protein hormones maintain homeostatic feedback control of basal (tonic), pulsatile GnRH, and LH secretion. The following is a description of this physiological interplay, which controls basal LH secretion from late estrus through early proestrus and the preovulatory surge pattern of this gonadotropin on late proestrus.

Luteinizing Hormone

Role of Ovarian Steroids

NEGATIVE FEEDBACK CONTROL OF LH SECRETION

In the intact female rat, the secretion of LH is low from late estrus to early proestrus due to the negative feedback provided by estradiol and progesterone secreted by the ovaries. This negative feedback was first recognized by Moore and Price in 1932.[217] Such inhibitory effects on LH secretion can be most easily demonstrated by removing the ovaries. Early observations using bioassay endpoints[104] showed that both pituitary and plasma levels of LH rise following gonadectomy of the rat. These observations were extended using the more sensitive radioimmunoassays. Plasma levels of LH rise slowly over a number of days following ovariectomy.[218–220] The earliest significant rise occurs by 18–24 h after ovariectomy, with diestrus being the most responsive day for removal of the inhibitory feedback response.[219,220]

As pointed out by Schwartz and McCormack,[104] the hundreds of attempts at restoring LH levels to pregonadectomy levels with estradiol and progesterone have presented varying experimental approaches, often without reference to the intact animal. It is now generally accepted that the most potent ovarian steroid for inhibiting LH secretion is estradiol.[104] In long-term ovariectomized rats, LH levels are significantly lowered by a single injection of as little as 0.2 μg of estradiol benzoate.[221] Progesterone administration alone is ineffective in lowering the elevated LH levels in ovariectomized rats.[220,222,223] However, administration of progesterone to estradiol-treated ovariectomized rats lowers LH levels below those of animals treated with estradiol alone.[222] Moreover, simulation of the basal levels of estradiol and progesterone secreted from metestrus through early diestrus prevents the postcastration rise of LH in animals ovariectomized on metestrus.[224] From these data, it can be inferred that the levels of estradiol and progesterone secreted from metestrus through diestrus are capable of restraining the hypothalamo–pituitary axis and leading to basal secretion of LH during this time. Now that the pulsatile manner of LH secretion is appreciated,[90–92] it is apparent that estradiol and progesterone differentially modify LH secretion by influencing the frequency and amplitude of the pulses. It is also apparent that these steroids affect pulse amplitude and frequency differently on the various days of the cycle as well as in long-term ovariectomized rats, in which estradiol suppresses mean LH levels by decreasing pulse frequency.[225] Using slightly different experimental approaches, both progesterone[226] and estradiol[227] have been assigned the role of inhibiting mean LH levels from metestrus through diestrus by suppressing pulse amplitude. During the interval from estrus to the morning of metestrus, however, mean LH increases slightly due to increased pulse amplitude and frequency which is independent of ovarian steroid negative feedback control.[228] It seems likely that the steroids exert their negative feedback on LH secretion by modifying the amplitude and frequency of the LH pulses. The effects on frequency and amplitude may be exerted at the hypothalamic level by directly or indirectly modifying the pulsatile release of GnRH into the hypophysial portal blood supply to the pituitary gland. Some negative feedback control of pulse amplitude also appear to be exerted at the pituitary level by suppression of the responsiveness of gonadotropes to pulses of GnRH.[229]

STIMULATION OF THE PREOVULATORY LH SURGE

There is voluminous literature indicating that ovarian estradiol, secreted on diestrus and the morning of proestrus, provides the positive feedback stimulus for the ovulation-inducing surge of LH. There are several experimental approaches that have attested to this fact. Specifically, under either a 14:10 or 12:12 light–dark cycle with noon as the midpoint of the light interval,[87,88,230] ovariectomized rats will respond to a dose of exogenous estradiol by generating a proestrous-like LH surge the following day with the same timing and duration as the intact rat.[231–239] However, the magnitude of this induced surge differs from the spontaneous surge on proestrus. That is, baseline values are not as low and peak values are not as high as those on proestrus. In addition, rather than secreting a surge of LH on the single day after estradiol administration, ovariectomized animals respond with initiation of LH surges at similar times on each of at least the following 3 days.[231–239] Thus, a daily neural event must be coupled with the presence of estradiol to eventuate in daily LH surges in the ovariectomized rat. This confirms Everett and Sawyer's[103] original proposal of a 24-h periodicity in the LH release apparatus. Other approaches have demonstrated that ovarian estradiol secreted on diestrus is required to activate the LH surge on proestrus. Specifically, administration of estrogen antagonists,[240] estradiol antiserum,[241,242] or ovariectomy[243] on diestrus blocks the proestrous surge of LH. On the other hand, administration of these agents or removal of the ovaries early on proestrus is ineffective in preventing this response later that same day. These data, taken together, suggest that the rising titer of estradiol secreted on diestrus is the major stimulus for the increased secretion of LH on proestrus.

The primary stimulus for the increased secretion of estradiol is the tonic pattern of LH and FSH secretion from metestrus through diestrus. Administration of sufficient antiserum to LH to neutralize tonic LH levels on diestrus-2 blocks the subsequent LH surge on proestrus.[244,245] Administration of that same dose of LH antiserum on early proestrus failed to block the LH surge late that afternoon.[244] LH antiserum preincubated with LH was without effect. Thus, the tonic levels of LH secreted on diestrus contribute to the stimulation of estradiol secretion by the ovarian follicle, which in turn stimulates the LH surge. It should also be noted here that tonic FSH secretion is also required to stimulate estradiol secretion, because an important action of this gonadotropin is to induce in granulosa cells the expression of aromatase, a critical enzyme responsible in the synthesis of estradiol from androgen that is produced by the theca cells in response to LH stimulation (see Chapter 28).

Unlike the long-term ovariectomized rat, which responds to a brief exposure to estradiol with daily LH surges, the intact rat presents LH surges only every 4–5 days in response to ovarian estradiol. Moreover, unlike the long-term ovariectomized rat, the acutely ovariectomized rat requires chronic exposure to estradiol to secrete daily LH surges.[237,238] Thus, some ovarian event associated with the LH surge on proestrus prevents expression of the daily neural signal eventuating in daily LH release. Indeed, it has been shown that

expression of the daily LH surge mechanism is limited to proestrus by the heightened secretion of progesterone on proestrus.[231,235] Interestingly, there is also every indication that the rise in progesterone on proestrus also facilitates the secretion of the surge of LH at this time. This idea first arose from Everett's observation in 1948[246] that injection of progesterone on the day prior to proestrus of a 5-day cycling rat would advance LH release and ovulation by 24 h. In fact, progesterone administered early on the morning of proestrus will advance LH release by several hours.[247] Similarly, progesterone administered to ovariectomized, estradiol-pretreated rats will induce an LH surge during the afternoon of that same day.[222] In addition, progesterone will enhance the magnitude of the estradiol-induced LH surge.[222] However, regardless of when injected, progesterone will not advance the LH surge beyond the early afternoon. Thus, LH release induced by progesterone is keyed to the time of day, not to the time the steroid was injected. Taken together, it seems that the proestrous surge of progesterone plays a dual role in controlling LH release during the cycle; specifically, it induces an initial early response that ensures the timeliness and increases the magnitude of the coincident LH surge induced by estradiol secretion during diestrus followed by a later extinction of the expression of the estradiol-induced 24-h periodic release of LH. At this time, there is no described role for the other major increase in progesterone secretion, which occurs from late metestrus through early diestrus, although it has been suggested that the duration of this secretion may determine whether a given cycle lasts 4 or 5 days.[248]

The stimulus for the increase in progesterone secretion on proestrus appears to be the proestrous surge of LH itself. Hypophysectomy on proestrus[72] or administration of pentobarbital to block the proestrous surge of LH[76,249] prevents the major ise in progesterone occurring later that day. Administration of LH reversed the effect of pentobarbital blockade of progesterone secretion.[76,249] Although the progesterone secreted on proestrus arises from the preovulatory ovarian follicle and is controlled by the proestrous surge of LH, the increase in progesterone from metestrus through diestrus is secreted ephemerally from the newly formed corpora lutea and appears to be autonomous of pituitary support. Indeed, Uchida and co-workers[72] found that hypophysectomy on estrus, shortly after corpus luteum formation, did not prevent the increase of progesterone on metestrus. Thus, this increase occurs independently of any continued pituitary luteotrophic support.

Interestingly, there appears to be a nonligand-dependent role for the progesterone receptor (PR) in the induction of a proestrus-like LH surge by estradiol.[250] Pharmacologic blockade of PR blocks the LH (and also primary FSH; see later) surge on proestrus in rats.[251–253] Ovariectomized PR knockout mice will not respond to an estradiol challenge or a self-priming paradigm of GnRH administration with significant LH secretion.[254] Peripheral administration of a PR antagonist blocks the estradiol-induced increase in GnRH levels in the ME and the proestrous surge of LH in plasma.[158] Moreover, implantation of an antisense PR oligonucleotide into the third ventricle prevented PR expression in the anteroventral periventricular region of the hypothalamus and blocked estradiol-induced LH surges.[158] Such PR activation requires cAMP.[255] Taken together, these data suggest expression of estradiol-induced LH surges requires ligand-independent PR activation. If this is the case, the precise role of the ligand, progesterone, must be more thoroughly evaluated. One possibility is that progesterone downregulates the activated PR to prevent LH surges on subsequent days.

STEROID RECEPTORS MEDIATING OVARIAN STEROID CONTROL OF LH DURING THE ESTROUS CYCLE

As discussed above, ovarian estradiol and progesterone secretions exert both negative and positive feedback actions in the brain and anterior pituitary gland, and thereby regulate the sequence of neuroendocrine events that in turn direct ovulatory cyclicity. The steroid receptors that convey these feedback actions have been identified largely through the study of the consequences of receptor gene knockout (KO) and knock-in (KI) in mice. Estrogen receptor α (ERα) knockout (ERαKO) mice exhibit hypergonadotropism and an absence of estrous cyclicity, and are refractory to both inhibitory and stimulatory actions of estradiol on LH secretion.[256–259] Null mutation of the ERβ isoform, by contrast, does not produce any such major defects[256] and studies to date have yet to document any major contributions of other physiological ERs to the regulation of gonadotropin secretion and control of the estrous cycle. Thus, ERα likely conveys most, if not all, of the negative and positive feedback actions of estradiol during the estrous cycle. Recent studies of tissue- and cell-specific ERα null mutant mice have also provided evidence that estradiol exerts feedback actions via ERα activation in both brain[259,260] and pituitary cells,[261,262] as discussed below. Moreover, the ERα signaling mechanisms that mediate steroid feedback actions have become clearer through the use of *ERα* gene mutant mice.

ERα can convey signals through classical genotropic pathways involving dimerization of ERα and binding to estrogen response elements in DNA to alter transcription of target genes. In nonclassical mechanisms, signaling proceeds in the absence of direct binding of ERα to DNA, through protein–protein interactions that alter transcription at non-ERE sites, or through signaling pathways activated by ERα complexed at the plasma membrane. A nonclassical ER knock-in (NERKI) mutant mouse has

been developed,[263] which isolates nonclassical ER signaling and permits analysis of the contributions that classical versus nonclassical signaling mechanisms may make to the manifestation of negative and positive feedback effects of estradiol during the estrous cycle. These studies have clearly demonstrated that classical ER signaling is an obligatory component of positive feedback regulation of LH secretion, while nonclassical mechanisms are sufficient for conveying the majority of the ERα-mediated negative feedback actions of estradiol on LH secretion.[258]

Progesterone receptor knockout (PRKO) mice have also been used to assess the importance of PRs in the regulation of cyclic LH secretion. The absence of estrous cyclicity and steroid feedback actions in these animals indicates that they play a primary role in the control of LH secretion during the estrous cycle.[254,264] The major importance of the PR_A isoform as opposed to the PR_B isoform in the control of LH secretion has also been confirmed in a study of PR isoform-specific KO mice 5.[265]

Sites at Which the Ovarian Steroids Act in Controlling LH Secretion during the Estrous Cycle

THE ANTERIOR PITUITARY AS A SITE FOR NEGATIVE FEEDBACK OF LH SECRETION BY OVARIAN STEROIDS

It is likely that the pituitary is a target for some of the inhibitory feedback effects of ovarian steroids on LH.[266,267] Ovariectomy increases[268,269] and estradiol decreases[266,269–271] the magnitude of LH released in response to GnRH administration, which suggests that the pituitary is indeed a locus of negative feedback by estradiol. These inhibitory actions can be observed within minutes following estradiol treatment, in contrast to the stimulatory actions of estradiol on LH secretion, which take 18–24 h to manifest. Although direct inhibitory effects of estradiol on LH secretion have been difficult to demonstrate using isolated anterior pituitary glands in vitro,[272] estradiol injections in vivo have been shown to suppress LH secretion from ectopic pituitary tissues exposed to chronic pulsatile GnRH stimulation.[229] Moreover, pituitary-specific ablation of ERα confers LH hypersecretion in female mice, indicating that some proportion of the negative feedback actions of estradiol occurs at the level of the pituitary, presumably the gonadotrope.[261,262] Increased serum LH levels in these mice are nevertheless much lower than levels noted in complete ERα knockout (ERαKO) mice, suggesting that the majority of the negative feedback actions of estradiol, or at least those mediated by ERα, are exerted at neural loci.

NEURAL SITES AT WHICH OVARIAN STEROIDS ACT TO INHIBIT LH SECRETION

Estradiol exerts central negative feedback actions, as first demonstrated by the finding that ovariectomy increases and estradiol treatment decreases GnRH concentrations in portal vessel blood of anesthetized female rats.[273,274] With the understanding that a neural GnRH pulse generator directs pulsatile LH secretion (see also Chapter 28), neural feedback actions of ovarian steroids were also inferred in studies documenting changes in the frequency of pulsatile LH secretion in peripheral blood. The frequency and amplitude of pulsatile LH secretion is increased following removal of the ovaries on metaestrus while replacement with estradiol and progesterone at physiological levels returns both parameters to ovary-intact levels.[226]

The brain has also been confirmed as a major locus of negative feedback mediated by ERα, as estradiol suppression of LH secretion was found to be absent in female mice bearing conditional deletion of ERα in neurons.[260] Confirmation of the specific sites of feedback in the brain has proven more challenging. Early studies used classic lesion[275] and steroid implantation[276] techniques in attempts to identify loci of these steroid negative feedback actions in the brain. In retrospect, this work was complicated by the inability of these approaches to discretely eliminate or activate specific neural networks without disabling the GnRH neurosecretory system itself, or impairing afferent networks required for its basic functioning. The persistence of estradiol negative feedback actions after complete hypothalamic deafferentation has been viewed as evidence that these effects are mediated by mediobasal hypothalamic neurons.[277] The complete and permanent separation of more rostral elements from the mediobasal hypothalamus must be questioned in these early studies, however, because LH pulsatility was found to continue in these animals, even after presumed transection of the majority of GnRH axons. At present, no studies have succeeded in identifying specific neuroanatomical locations containing cell populations that are necessary for conveying ovarian steroid negative feedback during the estrous cycle. The advent of cell-specific gene targeting in mice, however, is providing new clues as to the neurotransmitter cell phenotypes that may mediate some of these effects, as noted in subsequent sections.

THE ANTERIOR PITUITARY GLAND AS A SITE AT WHICH OVARIAN STEROIDS STIMULATE LH SECRETION

There is little doubt that the rising titers of estradiol through diestrus and the surge quantities of progesterone secreted on proestrus act at the gonadotrope to enhance its responsiveness to GnRH. Indeed, both basal and GnRH-stimulated LH release from cultured anterior pituitary cells are enhanced by the addition of estradiol to the culture medium.[278] Similarly, pituitary cells obtained during estradiol-dominated phases of the cycle are most responsive to GnRH.[279] In addition, within the first 3–8 h of introduction to the media, progesterone magnifies the estradiol-induced enhancement of the

LH-secretory response to GnRH.[280] However, by 48 h after introduction, progesterone significantly depresses the estradiol-induced enhanced LH secretory response to GnRH.[281] This effect of addition of steroids to pituitary cells in vitro mirrors the LH-secretory response to GnRH in vivo.[161] That is, from diestrus through early proestrus, rats secrete increasing amounts of estradiol and also respond to a challenge of GnRH with increasing maximal increments in plasma concentration of LH.

Coinciding with the proestrous surge of progesterone, the pituitary gland presents a larger acute increment in LH secretory response to GnRH, which is only present for approximately 2 h. After this time, the LH response to GnRH declines rapidly, reaching a nadir by the early morning of metestrus. These effects can be mimicked in vivo by appropriately timed administration of steroids to ovariectomized rats.[282] These data, and others, convincingly demonstrate that ovarian estradiol secreted from metestrus through early proestrus and ovarian progesterone secreted during the afternoon of proestrus sensitizes the pituitary gland to respond to GnRH. The later effects of the proestrous surge of progesterone are to blunt or completely inhibit the response to GnRH. These data suggest that the proestrous surge of LH is the consequence of two integrated actions brought about by the rising titers of estradiol from diestrus through proestrus: release of the GnRH surge on the afternoon of proestrous, and enhanced sensitivity of the pituitary gland to stimulation by the GnRH surge. A further increment in pituitary sensitivity is the result of the initial phases of the proestrous surge of progesterone. The relative insensitivity of the gland on estrus through diestrus may be the late response to the proestrous surge of progesterone.

Just how estradiol and progesterone modify cyclic response of the pituitary gland to GnRH is not known at this time. It had been rather widely suggested that the ovarian steroids enhance the pituitary response to GnRH by increasing GnRH receptor number,[262,283–285] while the diminished response induced by progesterone was a function of declining receptor number.[286] However, it now appears that the estrogen-induced heightened, or progesterone-regulated diminished, responsiveness may occur independent of a change in GnRH receptor number and may involve a postreceptor mechanism.[287–289] One likely postreceptor mechanism appears to involve the ability of estradiol to confer the capacity for gonadotropes to exhibit increasing LH secretory responses to successive pulses of GnRH—that is, the GnRH self-priming effect.[160] Estradiol may exert this permissive effect by inducing the expression of PRs in gonadotropes, which may then be activated by both ligand-dependent and ligand-independent mechanisms.[254,290] The latter appears to include activation of the PR_A isoform by GnRH receptor signaling via protein kinase A and/or protein kinase C–mediated pathways.[291] The mechanisms by which progesterone may thereafter exert delayed suppressive effects of on pituitary responsiveness remain unknown. Estradiol may also sensitize female rat pituitary cells to GnRH stimulation by regulating components of both PKC- and Ca^{2+}-dependent signaling pathways that are coupled to GnRH receptor activation.[292]

CONTROL OF LH SUBUNIT MESSAGE

Feedback actions of steroids may also be exerted through the regulation of gonadotropin subunit gene expression. There is little doubt that the gonads influence the expression of the LH subunit mRNAs. Ovariectomy dramatically enhances the expression of the α subunit mRNA and, to a lesser extent, LH-β subunit mRNA in the pituitary gland.[293] Moreover, treatment of ovariectomized rats with an estrogen and progesterone regimen that lowers serum levels of LH also lowers α subunit mRNA and LH-β subunit mRNA levels in the pituitary gland.[294] Ovariectomized rats treated with estradiol to induce a preovulatory-like surge of LH secretion presented a concomitant increase in α subunit mRNA without change in LH-β mRNA.[295] However, when rat pituitary fragments were incubated with estradiol for 2 or 6 h, LH-β subunit mRNA synthesis was increased.[296] LH-β gene promoter analysis in clonal LβT2 gonadotropes has revealed that estradiol may enhance GnRH-stimulated LH subunit expression through regulation of transcription of activators and repressors of the LHβ promoter.[297] These data suggest an additional subunit message control mechanism, wherein estradiol may differentially interact with frequency- or amplitude-modulated GnRH pulses to alter gonadotropin subunit gene expression. Indeed, in female rats, low-amplitude GnRH pulses elevate only LH-β subunit mRNA, whereas higher-amplitude pulses enhance both LH-β subunit and α subunit mRNAs.[296]

NEURAL SITES AT WHICH OVARIAN STEROIDS STIMULATE GnRH NEUROSECRETION

The evidence is overwhelming that estradiol acts at the diencephalon to stimulate release of a preovulatory GnRH surge, which in turn triggers secretion of an ovulation-inducing surge of LH on the afternoon of proestrus. As noted earlier, GnRH output by the MBH, as judged by push–pull perfusion, is pulsatile in nature with enhanced output prior to the expected surge of LH on proestrus.[102] Moreover, the concentrations of GnRH in hypophysial portal blood also increases dramatically just prior to the surge of LH on proestrus.[154] There is little doubt that the increased output of GnRH by the MBH is due to the enhanced secretion of estradiol on diestrus exciting peptidergic neurons in the preoptic-anterior hypothalamic area either directly or indirectly, to release a surge quantity of GnRH into portal blood.

That is, exogenous estradiol stimulates release of GnRH from neurovascular terminals in the ME, into hypophysial portal blood in a pattern equivalent to that secreted spontaneously on proestrus (Figure 26.12).[262] Most of the evidence points to rostral hypothalamic sites as the location for these stimulatory effects on GnRH secretion. Specifically, estradiol implanted directly into the POA-AHA elicits LH release,[262,298,299] while implantation of the ER antagonist, keoxifen, into the same area suppresses estradiol-induced LH surges.[300] Progesterone implanted in the preoptic area also exerts a stimulatory effect on LH secretion in estrogen-primed animals.[299] Implantation of steroids in other regions of the brain are largely without stimulatory effect on LH secretion. In addition, destruction of rostral inputs to the medial basal hypothalamus, while having no influence on the LH secretory response to ovariectomy or the negative feedback of ovarian steroids on LH secretion,[264] prevents the stimulatory effects of ovarian steroids on release of a surge-like pattern of LH secretion.[151,262,301–303] Also, direct implantation of progesterone within the mPOA blocked the expression of the 24-h periodic stimulus for release of a surge quantity of LH.[233] Placement of the steroid in other neural areas was ineffective. These and other data have served the argument that the LH "surge center" responsive to the positive feedback of estradiol and progesterone as well as the inhibitory effect of progesterone lies within the rostral hypothalamus.

Studies utilizing discrete lesions have more specifically implicated the AVPv as a critical target of the positive feedback actions of estradiol and progesterone.[139] Ablation of the AVPv renders animals incapable of maintaining estrous cycles or responding to LH surge-inducing regimens of estradiol and progesterone.[139] There is a sexual dimorphism in the volume of the AVPv in rats, with the larger nuclear phenotype in females[304] associated with the capacity of the female, but not the male, to respond to steroid feedback stimulation. The AVPv receives afferent inputs from the SCN that presumably convey a daily afternoon signal for release of the GnRH surge.[305] Vasopressin[306,307] and VIP[308,309] are among the neurotransmitters that likely mediate delivery of these circadian signals from the SCN to the AVPv. Estradiol appears to exert a major positive feedback action in AVPv neurons by coupling these afferent neural signals to the generation of efferent signals for the stimulation of the preovulatory GnRH surge. There is evidence that the cellular integrative mechanism that mediates this coupling process involves estradiol induction of PR expression in AVPv neurons, because knockdown of PR expression within the AVPv blocks steroid induction of the LH surge and treatment with the PR antagonist, RU486, blocks the release of GnRH and LH surges.[158,255] Moreover, estradiol treatments fail to evoke LH surges in PRKO mice,[254] revealing an absolute dependency of LH surge generation on the induction of PRs, most likely in the foregoing AVPv region.

It has been proposed that the daily neural signals conveyed by SCN afferents activate cAMP-PKA signaling pathways that initially prompt ligand-independent activation of PRs in AVPv neurons,[255] thereby activating AVPv neurons to deliver stimulatory signals via synaptic connections with GnRH neurons.[305] Additionally, the rising phase of the progesterone surge,[250] as well as estrogen-induced progesterone synthesis in hypothalamic astrocytes[310,311] likely contribute to the activation of these PRs and the amplification of GnRH surges. Supporting this idea are the observations that in vivo[312] and in vitro[313] measurements of GnRH release from hypothalamic tissues of adult ovariectomized rats treated with estrogen and progesterone in vivo results in enhanced release of GnRH relative to that from hypothalami of rats treated with estradiol alone. The expression of the immediate early gene product cFOS, which signifies neuronal activity, has been used to confirm that PR stimulation ultimately activates GnRH neurons in estradiol primed animals to a similar extent to that observed on the afternoon of proestrus.[314] Figure 26.13 depicts the sequence of events that are hypothesized to occur in AVPv neurons that culminate in the release of GnRH surges on the afternoon of proestrus. As described below, the phenotypes of AVPv neurons in which this integrative process occurs likely includes those that express kisspeptin.

Neurotransmitters and Neuropeptide Modulators Controlling GnRH and LH Secretion during the Estrous Cycle

The GnRH neuronal system functions as a final effector of neuroendocrine mechanisms that govern the reproductive axis and ovarian cyclicity. As such, it receives regulatory signals conveyed synaptically by afferent neural connections. Peripheral signals may also be directly registered by GnRH neurons, or these humoral cues may be delivered to the afferent circuitries that in turn regulate GnRH neuronal activity and secretion. Considerable progress has been made in identifying the neuronal cell groups that may integrate and communicate neural and peripheral signals to GnRH neurons, and thereby orchestrate the neuroendocrine control of ovulatory cyclicity. The advent of transgenesis and gene targeting approaches has facilitated this process, inasmuch as they have provided more definitive tests of the physiological relevance of mechanisms classically identified by pharmacological and anatomical approaches.

In general, neurotransmitters may contribute to the physiological control of estrous cyclicity by modulating basal pulsatile GnRH release, conveying synaptic signals that culminate in the release of the preovulatory

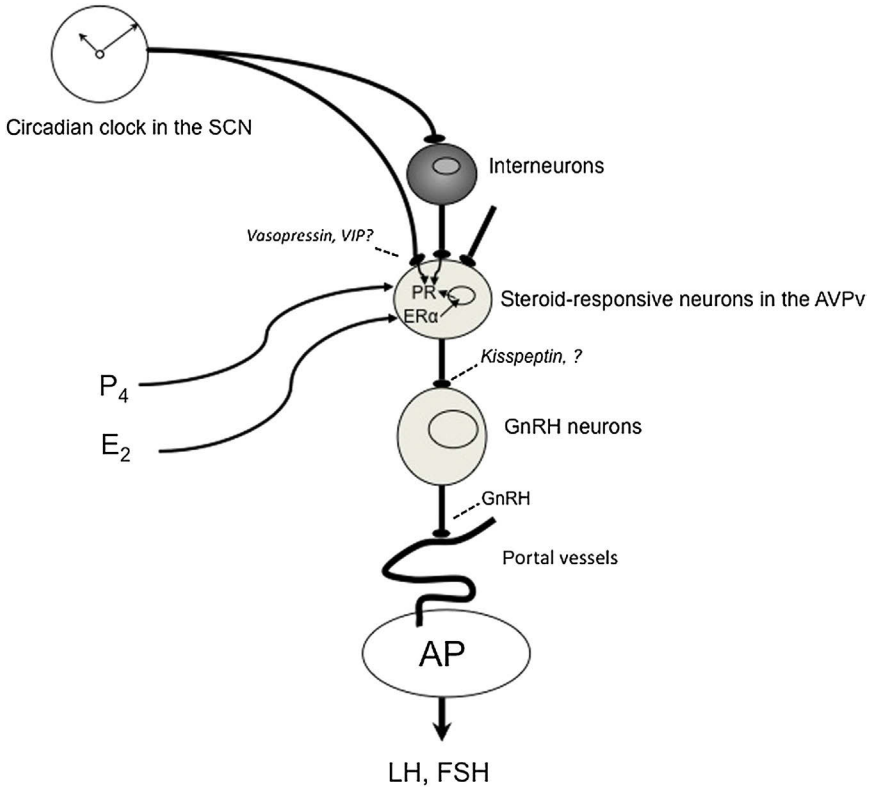

FIGURE 26.13 A model for the neuroendocrine integrative mechanisms that mediate release of preovulatory GnRH and gonadotropin surges. A daily neural signal is emitted on each afternoon of the estrous cycle from the master circadian clock in the suprachiasmatic nucleus (SCN), and conveyed to steroid-sensitive neurons in the anteroventral periventricular nucleus (AVPv). Vasopressin and vasoactive intestinal polypeptide (VIP) may function as neurotransmitters conveying the daily neural signal. In the absence of the preovulatory surge of estradiol (E_2), the signal is not coupled to the activation of AVPv neurons. The rising tide of E_2 on the evening of diestrus and morning of proestrus activates ERα in kisspeptin neurons and possibly other cells types in the AVPv, resulting in increased expression of progesterone (P_4) receptors (PRs) and other proteins that permit coupling of the incoming synaptic signals to the activation of the AVPv neurons. Excitation of AVPv kisspeptin neurons in turn stimulates release of the peptide at synapses on GnRH neurons, activating kisspeptin receptors and evoking excitation and release of a GnRH surge into the hypothalamic–hypophysial portal vasculature. The GnRH surge evokes an LH surge from the E_2-sensitized anterior pituitary (AP) gland. P_4 secretion evoked during the GnRH-induced LH surge functions to amplify the GnRH surge, and uncouple the AVPv neurons from the daily neural signal on the next day, effectively limiting the occurrence of the surge to the afternoon of proestrus.

GnRH surge, or simultaneously modulating both processes. Both positive and negative feedback regulation of GnRH release are believed to be exerted through actions in these afferent neurotransmitter cell groups, and not directly in GnRH neurons, because GnRH neurons do not to exhibit appreciable ER binding,[315] or detectable ERα protein.[316] The identities of those afferent neuronal populations that do express ERs, and thus may indirectly convey negative and positive feedback actions of the steroids to GnRH neurons, have remained the focus of intense research efforts for several decades. Below I note the neurotransmitter cell groups that have been most strongly implicated in the transmission of feedback actions of ovarian steroids, and more generally as modulators of either mode of GnRH release during the ovarian cycle. We focus in particular on neurotransmitter cell groups that express ERs and are known to function as monosynaptic modulators of GnRH neurons.

KISSPEPTIN

With the discovery that inactivating mutations of the kisspeptin receptor, KISS1R (also known as GPR54), are associated with hypogonadotropic hypogonadism in humans,[317,318] kisspeptin was identified in a variety of species as a powerful GnRH secretagogue that plays an important role in the regulation of pulsatile GnRH release in puberty, basal GnRH pulsatility in adulthood, and release of GnRH and LH surges during the estrous cycle (see also Chapters 11, 27, 28, and 30–32).[319,320] In rodents, kisspeptin-expressing neurons are concentrated in the ARN and in rostral periventricular regions that include the AVPv.[321] Co-expression of kisspeptin, neurokinin B, and dynorphin occurs within neurons of ARN,[322,323] prompting their designation as kisspeptin-neurokininB-dynorphin (KNDy) neurons.[324] Complete kisspeptin- or KISS1R deletion in transgenic mice results in infertility associated with hypogonadotropism and absent puberty and ovarian cyclicity in adulthood.[318,325–328] In *Kiss1R*

gene knockout mice, fertility can be completely restored by GnRH neuron-specific expression of KISS1R,[329] indicating that kisspeptin neurons serve as a final common afferent pathway controlling GnRH neurons and their neurosecretory activity during reproductive development and adult reproductive cycles.

Infusion of a kisspeptin receptor antagonist into the arcuate nucleus (ARN), but not the mPOA, retards GnRH pulse generation,[330] suggesting that ARN kisspeptin neurons and kisspeptin-KISS1R signaling function either as substrates of the GnRH pulse generator itself or as monosynaptic modulators of GnRH pulsatility.[329] One current hypothesis[322,324,331,332] holds that KNDy neurons function as an integrated and interconnected network, in which neurokinin B excites and dynorphin inhibits the activity of other KNDy neurons to produce and then transmit an intermittent, pulse-generating pattern of excitatory signals to GnRH neurons.

More than 90% of kisspeptin neurons in both the AVPv and ARN express ERα,[321] and thus it has been proposed that they play important roles in estradiol feedback regulation of GnRH release. Ovariectomy reduces and estradiol increases kisspeptin expression in the AVPv, whereas ovariectomy increases and estradiol reduces kisspeptin gene expression in the ARN.[333–337] It has therefore been hypothesized that estradiol exerts positive feedback through actions in AVPv kisspeptin neurons and negative feedback through effects on ARN kisspeptin neurons.[338] Evidence that AVPv neurons play a role in the generation of GnRH surges is compelling. Kisspeptin gene expression in the AVPv is increased at the time of the LH surge.[339] Localized passive immunoneutralization of kisspeptin in the POA, moreover, blocks the release of LH surges,[339,340] as does infusion of a KISS1R antagonist.[341] Estradiol treatments fail to stimulate kisspeptin gene expression in the AVPv of ERαKO mice,[333] consistent with the idea that ERα in these neurons mediates estradiol positive feedback actions. Observations of acyclicity and near absence of corpora lutea in kisspeptin cell-specific ERα knockout (KERKO) mice strongly suggest that ERα signaling in kisspeptin neurons is required for normal GnRH surge generation.[342] It remains to be determined if selective ablation of ERα in AVPv, but not in ARN, kisspeptin neurons produces the same result—a finding that would more specifically implicate AVPv kisspeptin neurons in the stimulation of GnRH surges. Nevertheless, the foregoing studies collectively provide strong support for the idea that kisspeptin neurons in the AVPv play an important role in conveying the positive feedback actions of estradiol.

A role for kisspeptin neurons in negative feedback has not yet been convincingly established. Consistent with this idea, nevertheless, is the observation that estradiol's negative feedback actions and its capacity to suppress kisspeptin gene expression in the ARN can both be mediated by nonclassical ERα signaling mechanisms.[333] In KERKO mice, elevated LH secretion occurs during the juvenile period, indicating that ERα in kisspeptin neurons mediates an estradiol negative feedback mechanism that functions to restrain GnRH release prior to puberty.[342] However, LH levels in KERKO mice decline to levels observed in wild-type animals during puberty, suggesting that a prepubertal ERα dependent negative feedback mechanism is supplanted in the adult by an ERα-, and perhaps kisspeptin, independent mechanism. It is not known if this reflects the normal activation of a postpubertal kisspeptin-independent negative feedback mechanism or if abnormal mechanisms are elicited in these animals to compensate for the absence of ERα signaling in kisspeptin neurons. The hypothesis that ARN neurons mediate steroid-negative feedback has not been supported by electrophysiological studies of identified kisspeptin neurons in hypothalamic slices from kisspeptin-GFP expressing transgenic mice; predictions that spontaneous firing of ARN kisspeptin neurons would be increased after ovariectomy and decreased with estradiol treatments have not been met.[343] Toxin-mediated ablation of KNDy neurons has been found to reduce the postovariectomy rise in LH secretion, but estradiol treatments of these animals were nevertheless effective in reducing serum LH to basal levels.[344] The latter effects may be due to the activity of 5% of KNDy neurons that survive the cell ablation procedures or to a pituitary site of action. Whether KNDy neurons are the site of estradiol negative feedback thus remains to be confirmed or refuted using methods for selective deletion or blockade of ERα in this discrete cell population in adult animals.

γ-AMINO BUTYRIC ACID

Hypothalamic cells that produce the neurotransmitter γ-amino butyric acid (GABA) also express ERs.[345] Thus, numerous studies have focused on their roles in regulating GnRH release during the estrous cycle and in response to steroid hormones. GABA has long been viewed as an inhibitory neurotransmitter in the brain, activating both postsynaptic ionotropic $GABA_A$ and presynaptic metabotropic $GABA_B$ receptors. Numerous GABA neurons are located in the AVPv, and expression of GABA transporters (VGAT) has been characterized in synaptic contacts on GnRH neurons;[346] many of these GABAergic neurons have also been shown to produce the excitatory amino acid neurotransmitter glutamate (see below).

Several in vivo studies have provided evidence supporting an inhibitory role for GABA in the regulation of GnRH release. GABA release, as measured by push–pull perfusion or microdialysis of the preoptic area, is reduced during estradiol-induced LH surges,[347–349] and GABA levels are increased during estradiol suppression of LH pulsatility.[350] Infusions of GABA into the preoptic area block the preovulatory LH surge in proestrous

rats,[351] whereas preoptic injections of a $GABA_A$ receptor agonist inhibit pulsatile LH secretion in ovariectomized rats.[352] However, blockade of $GABA_A$ receptors in the preoptic area by the receptor antagonist bicuculline also reduces, rather than stimulates, pulsatile LH secretion.[352] It was not found to have an effect on the LH surge in one study,[351] while another showed that it advanced its timing.[353] These contradictory results likely reflect the heterogeneaity of GABAergic neuronal influences on GnRH release that are difficult to parse with these in vivo, organ-level experimental manipulations.

More recent electrophysiological studies in tissue slices from GnRH-GFP transgenic mice have permitted direct and discrete analysis of direct synaptic regulation of GnRH neurons by afferent GABAergic neurons. Considerable evidence now supports the contention that GABAergic neurotransmission activates $GABA_A$ receptors in GnRH neurons to depolarize their membranes and increase the frequency of firing in these cells,[354–356] although some have questioned whether this is the predominant endogenous GABAergic control mechanism under all physiological and developmental circumstances.[357] Elevated chloride levels in GnRH neurons likely confers stimulatory responses to GABA through $GABA_A$ receptors, which function as chloride ion-selective pores. The stimulatory nature of this synaptic drive to GnRH neurons is supported by observations that the frequency of GABA neurotransmission and the postsynaptic responses of GnRH neurons to GABA in tissue slices are positively associated with GnRH neuron activity and LH release.[358–362] Recordings from GnRH neurons in an in vivo recording paradigm have provided a more complicated picture of GABAergic regulation of GnRH neurons, as responses to $GABA_A$ agonist are heterogeneous among GnRH neurons.[363] Nevertheless, $GABA_A$ receptor antagonism consistently reduces GnRH neuronal activation in this experimental paradigm. Surprisingly, partial GnRH neuron-specific knockdown of $GABA_A$ receptors does not impair ovulatory cyclicity, fecundity, or fertility[364]—a result that may indicate that few functional receptors are sufficient to mediate GABA effects or that one or more other synaptic inputs may compensate for the loss of $GABA_A$ mediated tone. See also Chapter 11 for further discussion of GABAergic regulation of the GnRH neuron.

Estradiol treatments that evoke daily LH surges evoke diurnal alterations in the frequency of GABA transmission to GnRH neurons, with reduced frequency in the morning correlated with negative feedback suppression of LH and increased frequency in the afternoon correlated with release of the GnRH surge.[365] These findings implicate GABAergic afferents in the transmission of both negative and positive feedback influences on GnRH release that operate before and then during the generation of preovulatory LH surges. It is not known at this time whether ERα in GABAergic neurons induces this synaptic signaling pattern, or if ERα signaling in higher-order afferents to GnRH neurons indirectly convey negative and positive feedback signals through these GABAergic interneurons. The locations of the GABA neurons that may function in this manner, and their functional relationship with kisspeptin neurons and other likely mediators of steroid hormone feedback actions, are not completely understood. Parallel kisspeptin and GABAergic pathways from the AVPv to GnRH neurons have been demonstrated and found to be sensitive to electrical stimulation of differing frequencies.[366] Other data demonstrate that kisspeptin increases GABA and glutamate (see below) transmission to GnRH neurons in an estradiol-dependent manner.[367] Thus, kisspeptin may influence GnRH neurons by multiple pathways, including direct actions via KISS1R signaling in GnRH neurons, as well as through modulation of fast GABAergic and glutamatergic neurotransmission to GnRH neurons.

GLUTAMATE

A considerable body of evidence exists that supports a stimulatory role for glutamate neurons in the regulation of GnRH release and ovulatory cyclicity.[368–370] Studies have implicated direct glutamatergic neurotransmission onto GnRH neurons in mediating these effects. GnRH neurons express both ionotropic glutamate receptors, the α-amino-3-hydro-5-methyl-4-isoxazole-propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors,[371–373] and postsynaptic currents mediated by receptors of both types have been recorded in GnRH neurons.[374] Peripheral and central administration of NMDA stimulates GnRH and LH release in a variety of species including the monkey,[375,376] and NMDA has been used as a reliable GnRH secretagogue in the study of puberty.[377] NMDA has also been shown to directly stimulate GnRH release from hypothalamic explants,[378] and spontaneous pulsatile GnRH release from hypothalamic tissues is suppressed by NMDA receptor blockade. In female rats, systemic challenge with NMDA evokes LH secretion in metestrus and proestrus rats but inhibits LH secretion in ovariectomized animals,[379] consistent with the findings that estradiol augments LH responses to NMDA stimulation.[380] One action of estradiol may therefore be to increase neuronal responsiveness to endogenous glutamate neurotransmission, possibly by increasing expression of receptors in GnRH neurons or other afferent circuitries. Glutamate release is increased in the preoptic area in association with estradiol- or estradiol and progesterone-induced LH surges[348,381] and antagonism of NMDA or AMPA receptors blocks estradiol-induced or preovulatory LH surges,[197,382,383] providing evidence that the positive feedback actions of steroid may also include stimulation of glutamate release.

Recordings of glutamatergic excitatory postsynaptic potentials (EPSCs) in GnRH cell bodies have not confirmed that glutamatergic activation of GnRH neurons is increased during the estradiol-induced LH surge,[374] although suppression of EPSC frequency was noted during the morning negative feedback phase. It is possible that increased glutamatergic neurotransmission may occur in dendritic synapses in GnRH neurons and would not be evident in this recording paradigm. Alternatively, estradiol may increase glutamatergic transmission at synapses on afferents to GnRH neurons. Further studies are required to more clearly characterize the cellular mechanisms by which estradiol may engage glutamate neurons in the service of feedback regulation of GnRH release.

MONOAMINES

The catecholamine neurotransmitters, principally norepinephrine (NE), have been known to be involved in the regulation of estrous cyclicity since the earliest days of neuroendocrine research.[384] The first indication was the demonstration that the α-adrenergic receptor antagonists dibenamine and SKF-501 blocked ovulation, presumably by preventing the release of an ovulation-inducing surge of LH.[262,385–387] Furthermore, blockade of the α-adrenergic postsynaptic receptor with chlorpromazine or depletion of catecholamines from presynaptic nerve terminals with reserpine also blocks ovulation.[388] Decades of subsequent research have provided evidence that NE and epinephrine-producing neurons in the brain stem are targets of ovarian hormone feedback signals, and can convey stimulatory synaptic signals for the release of GnRH and LH surges. For example, most catecholaminergic neurons in the caudal brainstem express the immediate early gene *cFos*, a correlate of excitation, coincident with the initiation of LH surges.[389] Subsets of these neurons also express ERα and send neural projections to preoptic and hypothalamic areas involved in surge generation.

Various measures of catecholamine turnover and release have been applied to assess the functional relationships between catecholaminergic neurotransmision in hypothalamic and preoptic areas and ovarian cyclicity. For example, with the semiquantitative microfluorometric assay, Lofstrom[390] observed that NE turnover is high in the subependymal layer in the ME of the rat during proestrus and low on all other days, while dopamine (DA) turnover rates in the lateral palisade zone of the ME are low on proestrus and high on other days of the cycle. While measuring the catecholamines by the more sensitive and specific radioenzymatic assay, Rance and co-workers[391] were able to thoroughly describe turnover rates of catecholamines in microdissected brain areas during the afternoon and early evening in proestrous rats. These were correlated with changes in GnRH concentrations in the ME and plasma concentrations of LH, FSH, PRL, estradiol, and progesterone. The concentrations of GnRH in the ME rose during the morning of proestrus (900–1200 h). At this time, plasma estradiol levels were elevated but both progesterone and FSH and LH remained basal. During this same time, both NE and DA turnover rates in the mPN, SCN, ARN, and the ME were low. Correlated with the beginning of the FSH and LH surges on proestrus, the ME concentrations of GnRH declined (1200–1400), while a significant rise in ME NE and DA turnover rates occurred. The decline in GnRH was probably a reflection of GnRH release into portal blood[154] and is correlated with the release of LH and FSH into peripheral plasma. During the time of enhanced GnRH, LH, and FSH release (1500–1700), turnover rates of NE in the ME remain elevated while those of DA decline. DA turnover rates also decline in the ARN but not the MPN. This decline in turnover of DA in the ME is correlated with enhanced release of pituitary PRL and is a reflection of the physiological inhibitory control the hypothalamus exerts over the secretion of PRL from the pituitary gland. Norepinephrine turnover rates remain elevated in the mPOA, SCN, and ARN and may be a reflection of the dynamic stimulatory control that NE exerts over the activity of GnRH neurons and subsequently the LH surge on proestrus.

In other studies, NE release in the POA has been monitored directly using push–pull perfusion[392] and more recently by microdialysis;[393] it was found to be increased in association with the initiation of LH surges. Moreover, selective lesioning of catecholaminergic terminals reduces LH surges, while reverse dialysis of NE stimulates LH secretion. Taken together, these findings reveal that caudal brain stem catecholaminergic neurons provide stimulatory signals for release of the LH surge, and hence for the progression of the estrous cycle. These signals may be modulatory in nature, however, and not obligatory for the release of LH surges; this idea is derived from the findings that depletion of hypothalamic catecholamines by transection of ascending noradrenergic pathways does not permanently impair ovarian cyclicity or release of LH surges.[394] That NE and epinephrine are not essential regulators of estrous cyclicity can also been inferred from the finding that dopamine β hydroxylase (*Dbh*) null mutant mice are fertile, and if treated during pregnancy with NE give birth to relatively normal progeny.[395] The caveat must be noted, however, that compensatory mechanisms may be engaged in both the foregoing pharmacological and gene targeting studies that obscure an essential role for NE in regulating ovulatory cyclicity under normal circumstances.

Most of the early data suggesting a role of the catecholamines in directly controlling GnRH release is inferential. That is, neurophysiological and neuropharmacologic manipulations within the diencephalon release LH, whereas application of pharmacologic agents directly

to the pituitary gland, in most cases, does not affect LH release. These data, along with a few direct measurements of GnRH in vitro[396] and in vivo,[397] have served to support the voluminous literature indicating that the catecholamines affect LH release by modifying the activity of GnRH neurons.[398] NE appears to be the most important of the catecholamines in controlling LH release. However, this catecholamine has a dual effect on LH release. Specifically, in the untreated ovariectomized rat, infusion of NE or adrenergic agonists into the third cerebral ventricle inhibits pulsatile LH release.[399,400] In addition, stimulation of the noradrenergic cell group in the locus coeruleus blocks the release of LH on proestrus.[401] Presumably, NE inhibits neurons in the mPOA responsible for pulsatile GnRH release into portal blood.[402] This inhibition is mediated by excitation of β-adrenergic receptors[403,404] since the effects can be blocked with propranolol, a β-blocker, but not phenoxybenzamine, an α-blocker. On the other hand, in ovariectomized rats in which serum LH was maintained at basal levels by ovarian steroids, NE or α- (but not β-) adrenergic agonists induced GnRH[405] and LH release.[400,406,407] Moreover, treatment with α-antagonists blocked the release of the preovulatory "surge" of LH on proestrus.[408] The noradrenergic fibers responsible for stimulation of GnRH and subsequently LH secretion also appear to arise from cell bodies in the locus coeruleus.[409–412] These data, taken together, suggest that LH release may be inhibited by a β-adrenergic mechanism in the preoptic area and stimulated via excitation of α-adrenergic receptors on GnRH neurons or unknown afferents to GnRH neurons.

Electrophysiological studies have revealed that the direct effect of NE on GnRH neurons is hyperpolarizing,[413] a response that occurs through both α1 adrenergic and β-adrenergic receptors in tissues obtained from ovarectomized and ovary-intact mice. These data suggest that noradrenergic facilitation of GnRH release during the ovulatory cycle is exerted indirectly, through synaptic intermediates to GnRH neurons. Clarification of the physiological roles of noradrenergic control of GnRH and LH secretion awaits further study in new rodent models that enable signaling analyses in complex, defined neural circuitries.

Older studies also implicated central epinephrine in the regulation of GnRH release.[398] For example, blockade of the proestrous surge of LH with pentobarbital[414] or LY78335, an inhibitor of phenylethanolamine-*N*-methyltransferase (PNMT; the enzyme that converts NE to epinephrine[415]), could only be reversed by administration of epinephrine in the cerebral ventricles. Administration of DA or NE was ineffective. Moreover, selective pharmacological suppression of epinephrine synthesis delayed the proestrus-like afternoon accumulation of GnRH in the ME and the resultant proestrus-like LH surge in ovariectomized estrogen–progesterone-treated rats.[416] These experiments, together with others, suggest a facilitating role for epinephrine in stimulating GnRH and subsequently LH release on the afternoon of proestrus. That *Dbh* gene knockout mice and *Pnmt* gene knockout mice are fertile[417] suggests that the role of epinephrine, like that ascribed to NE, is not an obligatory one in the regulation of estrous cycle.

Most of the literature provides an uncertain role for DA in control of the cyclic release of LH in the rat. DA turnover in the ME declines prior to the LH surge on proestrus.[391] Moreover, activation of central DA receptors inhibits the pulsatile release of LH.[418–420] These studies imply that DA may be a physiological inhibitor of GnRH and consequently LH release. However, DA has also been shown to stimulate GnRH release from ME fragments in vitro.[421] This suggests a stimulatory role for DA in cyclic LH release. Although it was shown that intraventricular injection of DA will cause LH release in ovariectomized steroid-treated rats,[422,423] this has not been confirmed in other laboratories.[400,406] Therefore, at the moment, a role for DA in control of the cyclic release of LH has not been unequivocally established. It is clear, nevertheless, that DA can exert powerful inhibitory effects on GnRH neuron excitabiity, and that these actions are mediated by both presynaptic and direct actions on GnRH neurons through both D1 and D2 receptor subtypes. It is possible that these effects serve to modulate both transmission and responsiveness to surge-induced signals from neurons in the AVPv.[424]

There are a large number of studies implicating 5-hydroxytryptamine (5-HT), or serotonin, derived from cell bodies in the dorsal and median raphe nuclei as both inhibitory or stimulatory to GnRH and consequent LH release. Enhancement of serotonin activity in regions of GnRH neurons along the preoptic-tuberal pathway depresses episodic LH release.[425–427] On the other hand, a large number of studies also indicate a stimulatory role for serotonin in the LH surge mechanism on proestrus. Indeed, the timing role of the SCN in the circadian nature of the LH release apparatus may be a function of its serotoninergic innervation from the raphe,[428–430] because there appears to be a diurnal rhythm of serotonin metabolism within the SCN.[428] Moreover, pharmacologic blockade of this rhythm disrupts the proestrous surge of LH[431] as well as the estrogen-induced daily surge of LH in ovariectomized rats.[430] However, lesions of the midbrain raphe, which reduce hypothalamic levels of serotonin, also diminish the estrogen-induced surge of LH in ovariectomized rats.[429,430] Taken together, these data imply that serotonin plays a dual role in release of LH: inhibitory to the GnRH pulse generator and stimulatory to the daily periodicity of the circadian oscillator. A study provided evidence that these dual actions of serotonin may be exerted in part by direct postsynaptic actions on GnRH neurons to regulate their electrical

excitability.[432] Thus, most GnRH neurons were found to exhibit a biphasic response to serotonin comprised of a rapid inhibition and slower excitation mediated respectively by the 5-HT1A and 5-HT2A subtypes of the serotonin resceptor. The physiological significance of this dual control remains to be clarified.

ENDOGENOUS OPIOID PEPTIDES

The endogenous opioids have been shown to play a physiological inhibitory role in the release of LH during the estrous cycle. Much of our understanding arises from the use of opioid receptor agonists such as morphine sulfate, analogs of the opiates, or opioid receptor antagonists such as naloxone or naltrexone. Morphine and the endogenous opioids block the proestrous surge of GnRH and subsequently LH,[433] as well as the LH surge induced by ovarian steroids in ovariectomized rats.[434,435] Early studies suggested that these effects may be due to the suppression of NE and epinephrine release from neurons that control GnRH secretion.[434,435] Through use of the μ opiate receptor blocker, naloxone, it is apparent that endogenous opioid tone is greatest on the days of metestrus, diestrus, and estrus and diminished immediately before and during the LH surge on proestrus.[436/437] In addition, the opiate tone is also reduced around the time of LH release in ovariectomized[438] or ovariectomized, steroid-treated rats.[437] These data, taken together, suggest that the endogenous opioids may play a physiologically significant restrictive role in control of LH secretion. The changes in opioid tone may be controlled, in turn, by the ovarian steroids.

Perhaps the most widely studied endogenous opioid peptide that may regulate LH secretion (and that of FSH and PRL) during the ovarian cycle is β-endorphin, which inhibits both pulsatile[439–441] and preovulatory[433–442] release of the gonadotrophins. β-endorphin, along with α-melanocyte-stimulating hormone (MSH) and other biologically active peptides, are derived via posttranslational proteolytic processing of the precursor protein, proopiomelantocortin (POMC), which is expressed at highest levels in the central nervous system (CNS) in a discrete neuronal population within the ARN. The POMC neurons in the ARN send projections to the rostral POA[443] and sites within the hypothalamus, through which they presumably act to suppress GnRH release in portal blood.[433,444] The actions of β-endorphin are mediated by μ opioid receptors[436,437] and may in part be exerted directly on GnRH neurons,[445] although one study failed to detect μ opioid receptor gene expression in GnRH cells.[446]

It has been hypothesized that POMC neurons convey negative feedback actions of estradiol during the ovulatory cycle because concentrations of the peptide in the POA, ARN, and ME are highest in diestrus and lowest in hours leading to the preovulatory GnRH and LH surges.[447] Estradiol can directly activate POMC neurons in hypothalamic tissue slices from guinea pigs, at least in part by desensitizing (uncoupling) $GABA_B$ receptors from their G-protein-gated inwardly rectifying K^+ channels.[448] A subset of POMC neurons expresses ERα, and POMC cell-specific deletion of these receptors results in reproductive deficits, including altered estrous cyclicity and reduced litter size.[449] Interestingly, however, estradiol failed to suppress LH secretion in these animals following ovariectomy, although it should be noted that postovariectomy LH levels were attenuated to the extent that suppression by the steroid may have not have been observable.

There is also evidence that prodynorphin-derived peptides, especially dynorphin A, may regulate GnRH and LH secretion on the afternoon of proestrus. Reduced expression and release of the peptides may serve to disinhibit GnRH neurons at the time of the proestrus surge,[450] as suggested by experiments in which infusions of the peptide were shown to block the LH surge, while local, passive immunoneutralization of the peptides in the MPOA advances the release of the LH surge.[451] These actions appear to be mediated by activation of kappa opioid peptide receptors.

RFAMIDE-RELATED PEPTIDE 3

RFRP-3, like kisspeptin, is a member of the arginine-phenylalanine-amide (RFamide) family of neuropeptides, but their effects on reproductive hormone secretions are diametrically opposed, with RFRP-3 inhibiting LH secretion. RFRP-3, which is the avian orthologue of GnIH identified by its ability to inhibit gonadotropin release from quail pituitaries in vitro,[452] is found in rodents to be produced by neurons located in the hypothalamic dorsomedial nucleus (DMN).[453] These neurons have been found to co-express ERα and form close appositions with GnRH neurons in hamsters.[453] In ovariectomized hamsters, systemic as well as intracerebroventricular injections of RFRP-3 rapidly suppress LH secretion, implying both central and pituitary actions of the peptide in this species. Similar studies in rats have not provided as clear a picture of the peptide's actions. One group has found that RFRP-3 decreases LH secretion when injected peripherally but not intraventricularly;[454] another found no effect of intravenous injections of RFRP-3 on serum LH.[455] Support for a central regulatory role for RFRP-3, however, comes from the observations that a subset of GnRH neurons express GPR74, a high-affinity receptor for the peptide, and that RFRP-3 exerts a direct postsynaptic inhibitory effect on GnRH neuron firing by activation of an inwardly rectifying K^+ conductance.[456,457] To date, there have been no direct tests of the hypothesis that RFRP-3 neurons directly or indirectly convey feedback effects of ovarian steroids on GnRH and LH secretion. That RFRP-3 neurons modulate a subset of kisspeptin cells in mice[455] adds to the possible scenarios by which this may occur.

NEUROPEPTIDE Y, OXYTOCIN, GALANIN, AND OTHER CENTRAL PEPTIDERGIC MODULATORS

A neurohumoral role for NPY, OT, and galanin in the control of LH secretion was identified above. Most of the literature shows that NPY enhances the LH and FSH-secretory responses to GnRH in vivo,[182,185,188,458–461] yet the data are equally compelling for a neuromodulatory role of this peptide. NPY stimulates the release of GnRH from the medial basal hypothalamus in vitro,[460] and synaptic contacts have been identified between GnRH and NPY-containing axonal fibers in the septo-POA.[462] LH surges are attenuated in NPY-knockout mice.[193] These neuromodulatory effects are probably mediated by Y1 receptors.[463] Estradiol induces NPY1 receptor expression through nonligand-dependent activation of PR.[464,465] The stimulatory actions of NPY on GnRH release are heightened on proestrous,[464] suggesting that the preovulatory surge of estradiol may upregulate responsiveness to NPY stimulation through Y1 receptors by stimulating expression of these receptors in the mediobasal hypothalamus. Taken together, the foregoing findings support a role for NPY and Y1 receptors in mediating a portion of the positive feedback action of estradiol.

Although the bulk of the evidence suggests that NPY exerts stimulatory effects on GnRH neurosecretion, there are also data to suggest that NPY may inhibit GnRH neurosecretion by acting centrally at a Y-5 receptor.[466] Thus, it appears quite likely that NPY enhances the surge mode of GnRH neuronal activity through Y-1 receptors yet inhibits basal GnRH neuronal activity through a Y-5 receptor. On the other hand, stimulation of basal GnRH release may be attributed to a Y-4 receptor.[467] Taken together, it seems that summoning the well-known link between undernutrition and the loss of fertility, the stimulation of NPY activity by negative energy balance may also lead to an inhibitory effect on GnRH neurons.[468,469] OT may also act as a neuromodulator. Central but not peripheral administration of an antiserum to OT blocks the proestrous surge of LH,[470] suggesting a neuromodulatory role for this peptide in the control of GnRH secretion. Excitatory effects of OT may occur at GnRH axon terminals,[471] stimulating the activity of nitric oxide synthase via NE and resulting in an increased release of nitric oxide, which increases prostaglandin-E2 release that in turn induces GnRH release.[472,473]

Galanin may also act as a neuromodulator because it stimulates NE release from hypothalamic slices.[474] Because galanin has been found to be an estrogen-inducible secretory product of the anterior pituitary,[475,476] it could subserve a paracrine/autocrine role in that gland.[477] Moreover, because galanin genes are expressed in hypothalamic GnRH-containing neurons, there is a possibility of autocrine regulation at the hypothalamic level.[478]

Other neuropeptides have been shown to affect release of LH under a variety of experimental conditions. However, to date, none have been shown to play a physiological role in control of cyclic release of LH. Those neuropeptides shown to inhibit LH release include arginine-vasotocin,[479] cholecystokinin,[480] neurotensin,[481] and gastrin.[482] The neuropeptides that have been shown to stimulate LH release include substance P.[481] In contrast, angiotensin II,[483,484] VIP[485–487] by a hypothalamic site of action on GnRH neurons,[308] and human pancreatic peptide[488] have been shown to have dual effects on LH secretion: the peptides are inhibitory in untreated ovariectomized animals and stimulatory in steroid-treated ovariectomized rats.

Circulating Orexigenic and Anorexigenic Factors Regulating the Estrous Cycle

Circulating adipocytokines, which were originally described for their orexigenic or satiety properties, have more recently been implicated in control of reproductive events such as puberty and the estrous cycle. It is not surprising that these peptides may have a critical role in reproduction because it has been well known that reproduction and reproductive development is optimally tied to feeding state. Although the adipocyte-derived satiety hormone, leptin, is not the primary signal that initiates the onset of puberty and estrous cyclicity, it acts in a permissive fashion, as a metabolic gate, to allow pubertal maturation to proceed if and when metabolic resources are deemed adequate.[489,490] In the adult, leptin stimulates LH, FSH, and PRL release in vitro[491] and in vivo.[492] Leptin also stimulates GnRH secretion in vivo[492] and in vitro.[493] This is not terribly surprising because leptin receptors have been found in the anterior pituitary gland,[494] in areas of the hypothalamus known to control secretion of these hormones,[495] and on hypothalamic neurons containing ERs.[496] However, the physiological significance of such observations should be tempered with caution because acute immunoneutralization of endogenous leptin in circulation is without effect on the proestrous as well as the estrogen/progesterone-induced surges of LH and PRL.[497] On the other hand, cycling rats treated with leptin antiserum for 8 days become anestrus.[498]

Studies have demonstrated that the ventral premammillary nucleus functions as an important target for leptin in the manifestation of its stimulatory effects on reproduction.[499] Lesions of this area disrupt estrous cycles, reduce LH levels, and prevent the ability of leptin to restore LH secretion during fasting. Moreover, re-expression of leptin receptors in the ventral premammillary nucleus is sufficient to induce puberty and improve fertility in female leptin receptor knockout mice.[500]

Central orexigenic neuropeptides also affect reproductive cyclicity. The orexins,[501] also known as

hypocretins,[502] are cleaved from a common precursor, prepro-orexin (130 aa residues), to form orexin A (residues 33–36; hypocretin 1 28–66) and orexin B (residues 69–96; hypocretin 2 residues 69–97). Orexin receptors are widely distributed throughout the hypothalamus. A subtype of the receptor, orexin 1, is found on GnRH neuronal cell bodies in the hypothalamus but not on GnRH terminals in the ME.[503] Orexin A fibers are also found in close apposition to 75–85% of these GnRH cell bodies.[503] Orexin A peptide is a potent stimulator of GnRH release from hypothalamic explants obtained from proestrous rats with diminished effects on explants obtained at other stages of the estrous cycle.[504] Similarly, intracerebroventricular injection of orexin A into estrogen/progesterone-treated ovariectomized rats, stimulates LH release in a dose-dependent manner.[505] However, the actions of orexin A on LH release may be site-specific. Indeed, injection of the peptide into the rostral preoptic area stimulates LH secretion; injection into the mPOA or the ARN/ME area inhibits LH secretion in ovariectomized estrogen/progesterone-treated rats.[506] Finally, hypothalamic content of orexin A is greater on proestrus than any other day of the estrous cycle in rats.[507] These data, taken together, suggest a potentially important role for the orexins in regulating the ovarian cycle of the rat.

The Gas Nitric Oxide as a Possible Regulator of the Estrous Cycle

Though the role of peptide and amine neurotransmitters in the regulation of the estrous cycle have been appreciated for some time, it is only recently that the neuroendocrine community has found that the gas nitric oxide (NO) plays a role as a neurotransmitter regulating the hypothalamo–pituitary axis. Unlike classical peptide and amine neurotransmitters, NO is not released from synaptic vesicles, does not interact with discrete receptor proteins, and does not function exclusively at synapses. NO is synthesized from the substrate L-arginine catalyzed by the enzyme nitric oxide synthase (NOS). There are three major isoforms of NOS that have been identified: neuronal NOS (n-NOS or type 1), macrophage NOS (m-NOS or type 2), and endothelial NOS (e-NOS or type 3).[508] n-NOS and e-NOS are constitutive, calcium- and calmodulin-dependent enzymes; m-NOS is an inducible calcium- and calmodulin-dependent enzyme. NOS has been found in the PVN, SON, OVLT, mPOA, SCN, ME, and ARN.[509–513] However, n-NOS is not co-localized in GnRH neurons obtained from rats[514,515] but is found in neurons bearing NMDA receptors.[509] Blockade of NO synthesis suppresses the LH surge in ovariectomized, steroid-treated rats,[516] and stimulation of NO release in cultured mediobasal hypothalamic tissues increases GnRH release.[517] However, stimulation of NO release in the POA suppresses GnRH neuronal firing,[518] indicating that NO effects on GnRH secretion are site specific.

Currently, it is believed that NO modulates GnRH release indirectly by influencing the GnRH secretory response to a host of stimulatory factors. These include the mediation of the GnRH secretory response to estradiol,[516,517,519–522] NPY,[523–525] and leptin.[526,527] Finally, NO has been found in gonadotrophs of the anterior pituitary gland[528] and is stimulated[529] or inhibited[530] by estradiol and stimulated by GnRH.[531] A precise role for NO of pituitary origin has yet to be described.

Growth Factors as Possible Regulators of the Estrous Cycle

Among the growth factors, epidermal growth factor (EGF), insulin-like growth factors (IGF), and nerve growth factor (NGF) may be implicated in the neuroendocrine control of the ovarian cycle of the rat.[532] Much of the data suggest a supportive role in the facilitation of LH and FSH secretion. Prior to the preovulatory release of LH during the late afternoon of proestrus, there is a significant increase in the proportion of gonadotrophs in the anterior lobe of the pituitary gland.[533–536] During this same period, there is an increased expression of EGF and EGF receptor on gonadotrophs to reach a peak on the afternoon of proestrus.[537,538] Both EGF itself and GnRH independently increased the expression of EGF receptor on gonadotrophs.[532] In addition to its mitogenic effect,[539] EGF may also be responsible for differentiation of gonadotrophs bearing GnRH receptors.[540,541] EGF release from gonadotrophs appears to be controlled by estradiol[542] as well as GnRH.[542] Taken together, it seems that EGF subserves an important autocrine or paracrine role by increasing the proportion of gonadotrophs responsive to GnRH, which will produce the ovulation-inducing surge of LH.

Studies have suggested that IGF-1 and the IGF-1 receptor may serve an important role in coordinating somatic development with the pubertal initiation of cyclicity and its maintenance in adulthood. Mice with the deletion of the IGF-1 receptor, but not the insulin receptor, in GnRH neurons exhibit delayed pubertal development with normal fertility. Administration of IGF1 advanced puberty in normal females but was ineffective in the GnRH-IGF-1 receptor knockout mice.[543] Blockade of IGF-1 receptors attenuates steroid-induced LH surges and reduces the percentage of GnRH neurons expressing cFOS in the preoptic area.[544] Long-term IGF-1 gene therapy in the MBH preserves estrous cyclicity in middle-aged female rats, suggesting that this growth factor may additionally play a role in the maintenance of adult GnRH neuronal function and protection against premature aging associated declines in activity.[545] During the estrous cycle of the rat, the number of synaptic

inputs to neuronal somas in the ARN decreases between the morning of proestrus and the morning of estrus. This decline in synaptic input and the accompanying increase in glial ensheathing of neuronal somas are blocked by an IGF-I receptor antagonist. In contrast, the IGF-I receptor antagonist did not affect the basal number of synapses or the morphology of synaptic terminals or length of the synaptic contacts. These findings indicate that IGF-I receptor activation may be involved in the phasic remodeling of ARN synapses during the estrous cycle.[546] Both IGF-I and IGF-II may additionally regulate LH secretion at the level of the pituitary gland, as both enhance basal and GnRH-stimulated LH release in vitro.[547–549]

Nerve growth factor (NGF) is a 26-kd protein found colocalized in virtually every cell of the anterior pituitary gland[550–553] and secreted.[550] Many of these same cells also contain the high affinity form of the NGF receptor.[551] NGF is also found in the ovary[554] as well as in the CNS.[555] However, the levels of the protein or the message do not appear to differ throughout the estrous cycle of the rat.[555] Although there are no data to suggest a direct neuroendocrine control of the ovarian cycle, its importance may lie in a potential role in differentiation of somatomammotrophs into mammotrophs.[552,556,557]

Follicle-Stimulating Hormone

It is apparent from the earlier discussion (Patterns of Pituitary Hormone Secretion and mRNA Levels during the Estrous Cycle) that the pattern of secretion of FSH bears some basic similarities to that of LH. The basic differences are the bimodal pattern of FSH secretion on proestrus through early estrus[52,62,85,89] and the longer interpulse interval of FSH than LH in gonadectomized animals.[95] It will be apparent from the following discussion that these differences may be attributable to differential control of FSH and LH secretion by the hypothalamus as well as by ovarian steroidal and nonsteroidal hormones. Because the common control of basal FSH and LH secretion by GnRH has been discussed, most attention is focused on the ovarian factors that regulate FSH secretion and their potential interaction with GnRH stimulation.

Role of Ovarian Steroid and Nonsteroidal Hormones Secreted Throughout the Estrous Cycle

The similarities in control of FSH and LH secretion by ovarian steroids abound. For example, just as the most potent negative feedback for LH is mediated by estradiol secreted from late estrus through early proestrus, the same relationship obtains for inhibition of FSH secretion.[104] Moreover, secretion of progesterone during this same interval potentiates the inhibitory effect of estrogen on secretion of FSH. However, not all of the inhibition of FSH secretion can be attributed to the ovarian steroids. Much of the physiological literature favors a major role for inhibin, a protein hormone originally designated as such in the male[558] and as folliculostatin[559] in the female. The hormone is primarily synthesized by the granulosa cells of the ovarian follicle[560] and appears in high concentration in follicular fluid. In fact, porcine follicular fluid (pFF) orignially served as a reliable source of inhibin bioactivity. In addition, the bioactivity or immunoreactivity is found in ovarian venous plasma,[561–563] the corpus luteum of pseudopregnancy[564] and the anterior pituitary gland within the gonadotroph[565] of the rat.

The initial evidence for the existence of a nonsteroidal ovarian factor regulating cyclic FSH secretion was indirect. Simulation of the patterns of estradiol and progesterone circulating throughout the estrous cycle are

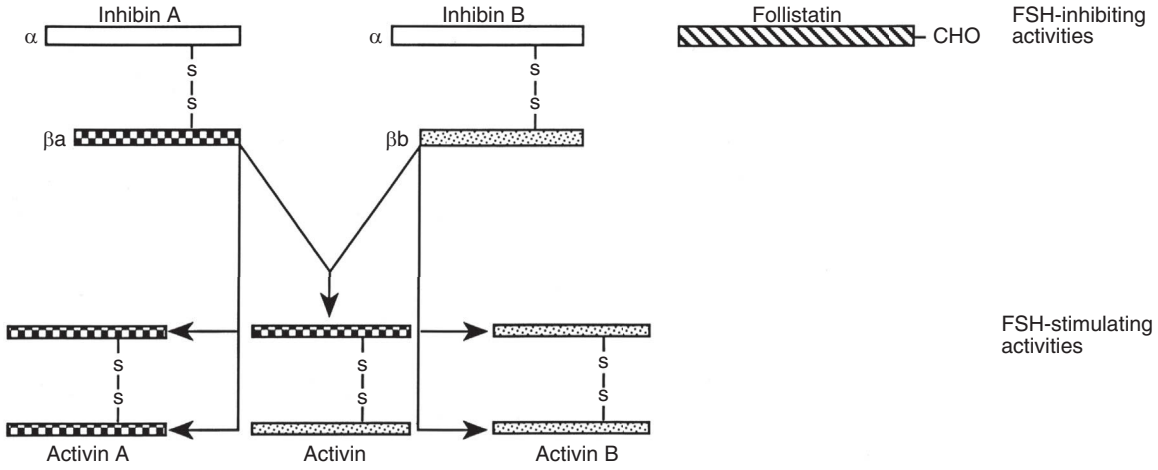

FIGURE 26.14 Diagrammatic representation of the three FSH-inhibiting proteins (inhibin A, inhibin B, follistatin) and three FSH-stimulating proteins (activin AB, activin A, activin B) found in the gonads of the rat. Similar patterned bars represent similar amino acid sequences in the respective subunits. Arrows point to proposed structural relationships between the inhibins and activins. See text for details.

sufficient to maintain basal and induce surge quantities of LH in ovariectomized rats.[566] However, both basal levels and surge concentrations of FSH are 2–3 times higher in these steroid-treated ovariectomized rats than comparable secretory states in intact cycling animals.[566] Such observations argued for another ovarian factor that selectively inhibits excessive secretion of FSH and thus prevents uncontrolled follicular maturation. When administration of inhibin-containing porcine follicular fluid was superimposed upon the steroid treatments, both basal and peak concentrations of FSH were lowered to levels similar to those seen in intact cycling rats.[566] Interestingly, there was an inverse relationship between the amount of inhibin-like bioactivity in ovarian venous plasma and the secretion of FSH throughout the estrous cycle.[563] That is, during periods of basal secretion of FSH, inhibin-like activity in ovarian venous plasma is high; during the proestrous-estrous surge of FSH, the activity declines.

The activity of pFF attributed to inhibin was originally isolated and partially characterized as a 32-kd protein of 2 chains of 18 (designated as the α-subunit) and 14 kd (the β-subunit), respectively.[567] Two forms of 32-kd inhibin, designated inhibin A and B (Figure 26.14), were actually isolated.[567] The two forms share the same α-subunit but their β-subunits, though similar, are distinguishable by N-terminal microsequencing (Figure 26.14). The cDNA clones of the α- and β-subunits of inhibins A and B were isolated from a porcine cDNA library and the N-terminal sequences of α-, βA-, and βB subunits used to design oligonucleotide probes.[568] The three rat inhibin subunits were subsequently isolated using the porcine cDNA clones.[569,570] The inhibins of the various species are highly conserved. During the course of purification of inhibin, two separate and distinct FSH-regulating activities were also found.[567] One of these, designated activin (Figure 26.14), showed potent FSH-releasing properties in the inhibin bioassay.[567] When the amino acid sequence of the inhibins was deduced, it was found that they bore striking similarities to TGF-β.[568] Although TGF-β will enhance FSH release in vitro,[571] the FSH-releasing activities in the preparation were found to be dimers of the β subunits of the inhibins (Figure 26.14).

Activin A and B are dimers of βA and βB, respectively[572] Activins are expressed in most tissues, including the pituitary gland, and act via local paracrine/autocrine actions to effect a variety of developmental and cellular processes.[572] In the pituitary gland, the major actions of activin include the stimulation of FSHβ gene expression and FSH secretion. A third FSH-inhibitory activity distinct from the inhibins was also isolated from pFF (Figure 26.14) during the original search for inhibin.[567] This single-chain glycosylated polypeptide has an MW of 35-kd and was designated follistatin.[573] It was subsequently shown that follistatin exists as 32, 35, and 39-kd glycoproteins,[574] the varying forms accounted for by varying degrees of glycosylation. Follistatins are extracellular binding proteins that function to sequester, and hence inactivate, activins and thereby inhibit FSH release from rat pituitary cells in vitro.[575] In general, follistatin is produced locally, whereas inhibin functions as an ovarian feedback signal, to counterregulate the stimulatory actions of activins on FSH synthesis in and secretion from pituitary gonadotropes.

Like other ligands in the TGFβ family, activins signal through binding to a type II receptor, which recruits and phosphorylates a type I receptor. The type I receptor then phosphorylates receptor-regulated signaling molecules called SMADs[576,577] and other downstream pathways that convey signals ultimately regulating gene transcription and cell function. Activin signals are transmitted through two type II receptors, ActRII or ActRIIB, and one type I receptor, ActRIB/ALK4.[576,577] Beta-glycan functions as an inhibin co-receptor which, when bound by inhibin, induces the formation of ternary complex with the ActRII or ActRIIB, and thereby prevents signaling by activins.[578] Circulating and local inhibins act through this mechanism, in parallel with the local effects of follistatin, to antagonize activins stimulatory effects on FSHβ gene expression and FSH release. These actions appear to be of major importance in differentially regulating FSH production during the estrous cycle.

Much of what we have learned about inhibin, activin, and follistatin actions in vivo[572] is due to the purification of reasonable quantities of these materials as well as the advent of recombinant technology. Using recombinant technologies, the peptides can be synthesized and used for biological studies as well as antibodies produced for immunoneutralization or radioimmunoassay. Recombinant inhibin will suppress FSH but not LH release in ovariectomized rats[579] but suppress both LH and FSH release on proestrus without an impact on ovulation rate 5 days later.[580] Similarly, recombinant activin A[581] or purified activin A[582] stimulates FSH synthesis and secretion in vivo,[581] stabilizes[583] and enhances[581] FSH-β mRNA in vitro, and enhances estradiol secretion in hypophysectomized rats.[582] Thus, activin A may not only stimulate FSH secretion, but it may also play an autocrine or paracrine role in controlling ovarian estradiol secretion. Administration of antisera to the inhibin preparations not only enhances FSH release during the estrous cycle but also increases ovulation rate during the next estrous cycle.[584,585] Measurements of immunoreactive inhibin-α subunit in peripheral plasma throughout the estrous cycle of the rat[584,586,587] confirms the measurements of bioactive inhibin in ovarian venous plasma.[563]

Serum concentrations of inhibin are high during the morning of proestrus and then decline sharply to the lowest levels by 24:00 h on proestrus after the preovulatory LH and FSH surges. Peripheral plasma levels of inhibin then returned to peak levels by 15:00 h on the afternoon of estrus from which they again rapidly declined to another low level by 24:00 h on estrus. Thus, serum levels of immunoreactive inhibin are inversely correlated with those of FSH.[52] Although both inhibin A and inhibin B are inversely correlated to FSH, they are discordant during the follicular phase of the cycle; inhibin A is low on the morning of metestrus and rises steadily to a peak on proestrus, while inhibin B is elevated elevated on the mornings of metestrus, diestrus, and proestrus. The differing patterns of inhibin A and inhibin B during the period of follicular development on metestrus and diestrus suggest different follicle sources or regulation of these molecules during this period.[588]

The estradiol-induced proestrous surge of GnRH is responsible for increased pituitary follistatin gene expression that is associated with the primary FSH surge on proestrus.[589–590] At the time of the trough between the primary and secondary surge of FSH on proestrus (Figure 26.2), there is decline in pituitary follistatin, an increase in free circulating activin A and pituitary content of FSH-β mRNA,[565] and a fall in ciruclating inhibin concentrations. These three events lead to the subsequent secondary surge of FSH secretion.

Much of the in vitro evidence points to a stimulatory effect of ovarian steroids on FSH secretion directly. Estradiol appears to stimulate LH and FSH secretion by acting on the pituitary directly.[281] Moreover, progesterone potentiates this stimulatory effect of estradiol on gonadotrophin secretion.[281] However, unlike its direct stimulatory effect on LH secretion, the apparent effect of progesterone on FSH secretion may be secondary to its stimulatory effect on FSH synthesis.[281]

Interrelationship of Steroid and Nonsteroidal Hormones and GnRH in Controlling the Cyclic Release of FSH

As previously described, the rising titers of estradiol by early diestrus have excited specific neurons within the hypothalamus to release a surge of GnRH into the portal vasculature during the afternoon of proestrus.[109,272] Concentrations of GnRH reach an initial peak in the late afternoon hours on proestrus, begin to decline and then reach a second lower peak at 0200 h on the morning of estrus. The second peak of GnRH release was originally proposed as a signal for release of the secondary phase of FSH secretion on estrus.[154] However, the selective release of FSH in the absence of continued LH secretion strongly suggests that GnRH-independent mechanisms must predominate in the regulation of the secondary FSH surge. Indeed, several lines of evidence suggest that the secondary (estrus) phase of FSH secretion may be independent of hypothalamic support. When spontaneous FSH release on proestrus is blocked with phenobarbital, infusion of GnRH reinstitutes the proestrous phase of FSH release.[591] Moreover, administration of an antiserum to,[204] or an antagonist of, GnRH on the early afternoon of proestrus[206,592] was effective in blocking the proestrous phase of FSH release. However, these same treatments were ineffective in preventing the estrous phase of FSH release when given on late proestrus. In addition, complete hypothalamic deafferentation on late proestrus does not prevent the estrus phase of FSH release.[593] Such data emphasize the fact that the proestrous phase of FSH secretion is dependent on direct hypothalamic stimulation while the estrous phase may be independent of direct support.

The secondary phase of FSH secretion, however, can be eliminated by blockade of the primary gonadotropin surge.[594] This observation suggested that the secondary phase of FSH secretion on estrus results from the ability of the primary gonadotropin surge to trigger an acute alteration in the selective ovarian feedback regulation of FSH secretion. It is now known that the secondary phase of FSH secretion is mediated in large part as a consequence of the withdrawal of negative feedback regulation by ovarian inhibin, which in turn appears to be dependent upon the release of the primary gonadotropin surge. Thus, the preovulatory surge of LH directs a precipitous drop in inhibin production by inducing transcriptional inhibitors of inhibin α in granulosa cells.[594] The acute reduction in serum inhibin, together with a drop in local follistatin production in the pituitary gland, likely disinhibits activin signaling and hence increases FSHβ expression and FSH secretion. Schwartz and colleagues have also provided evidence that progesterone and/or PR activation in pituitary gonadotropes may also contribute to the selective release of FSH on estrous.[595]

The additional contribution of a neurohormonal stimulator of FSH secretion during the estrous cycle, independent of GnRH, remains possible. The identity of separate FSH-releasing factor has been sought for decades, yet as of the writing of this review no such hypophysiotropic factor has been found.

Prolactin

As noted earlier, the pattern of secretion of PRL during the estrous cycle is quite similar to that of LH (Figure 26.2). That is, it is secreted in low basal amounts for the major portion of the cycle except for a "surge" quantity on the afternoon of proestrus. This surge begins approximately 2 h prior to the preovulatory surge of LH on proestrus but reaches peak values coincident with that of LH on proestrus. Enumeration

of the similarities and differences in controls of the two surges follow.

Role of Ovarian Steroids in Control of the Cyclic Secretion of Prolactin

There is overwhelming literature which clearly states that the dominant and perhaps sole stimulus for the proestrous surge of PRL is the rising titer of estrogen on diestrus through early proestrus. Indeed, just as neutralization of the circulating levels of estradiol with an antiserum blocks the proestrous surge of LH, it also blocks the proestrous surge of PRL.[242] Similarly, just as estrogen will induce daily afternoon surges of LH in ovariectomized rats, it will also stimulate daily surges of PRL.[239] These data, taken together, clearly show that the proestrous surges of LH and PRL are both linked to a 24-h clock wherein expression is closely linked to the presence of estradiol. Moreover, progesterone administered on diestrus-3 of a 5-day cycle will advance the proestrous LH[246] and PRL[596] surges 24 h. However, unlike the same effects on LH, progesterone neither enhances magnitude of estrogen-induced PRL surges nor extinguishes the estrogen-induced diurnal rhythm of PRL release (M. E. Freeman, unpublished). Although estrogen administration will elevate basal levels of plasma PRL in male rats, surge levels of PRL do not result from such administration.[239] Such a sexually differential response results from androgenization of the hypothalamo–pituitary axis of the males during neonatal life[239] (see also Chapter 47). It is not known if the larger AVPv in the female is functionally linked to the capacity for estradiol-induced PRLsurges, as it is for estradiol-induced LH surges.

Sites and Mechanisms Whereby Ovarian Steroids Control Prolactin Secretion during the Estrous Cycle

There is little doubt that one locus for the stimulatory action of estradiol is the lactotroph cell of the pituitary gland. Indeed, incubation of enzymatically dissociated pituitary cells with estradiol enhances both the basal- and TRH-stimulated release of PRL[597] Moreover, co-incubation with progesterone prevents the stimulatory effect of estradiol while progesterone alone exerts no effect.[597] Estradiol exerts its excitatory effect in part by blocking the inhibitory influence of DA. The DA agonist, CB-154, is less effective in inhibiting basal or TRH-stimulated PRL secretion in the presence of estradiol.[597] This antidopaminergic action of estradiol is probably not due to antagonism of the DA receptor[597] but may act at a postreceptor calcium- or cyclic AMP-dependent step.[597,598] In a sense, estradiol functions as a PRL responsiveness factor; decreasing responsiveness to DA and allowing some spontaneous PRL release as well as increasing responsiveness to TRH.[599] The molecular mechanisms that mediate these effects of estradiol during the estrous cycle are not fully known. On the day of proestrus, but not on other days of the cycle, DA activates an inwardly rectifying K+ current that rapidly hyperpolarizes the lactotroph membrane,[600] due to activation of G protein-coupled inwardly rectifying K+ channels (GIRKs). It has been hypothesized that the preovulatory estradiol surge confers signaling through this mechanism on this day, thereby engendering a PRL secretory rebound in association with the abrupt reduction of DA concentrations in portal blood on the afternoon of proestrus. Estradiol additionally stimulates PRL gene expression, likely through an ERα-mediated mechanism.[601]

Estradiol also stimulates the proestrous surge of PRL by acting at the hypothalamic level. Earlier studies revealed that a cut placed immediately behind the optic chiasm blocked the induction of a proestrous-like surge of PRL by exogenous estradiol.[602] Because this retrochiasmatic cut transected caudal efferents from the mPOA as well as the SCN, and the latter is a timing regulator,[105] one cannot be absolutely certain, on the basis of transection studies, of the exact site of estrogen action in inducing the proestrous surge of PRL. It is more certain, however, on the basis of focal lesion as well as localized implantation studies, that both the mPOA and the SCN play a role. For example, lesions of the mPOA prevented the estrogen-dependent proestrus surge of PRL[603] and the estrogen-induced afternoon surge of PRL in ovariectomized rats.[604] In addition, lesions of the SCN blocked the estrogen-induced afternoon surge of PRL in ovariectomized rats.[605–607] These data suggest that both the MPOA and the SCN are involved in the cyclic PRL secretory response to ovarian estradiol. They do not indicate if one or both sites respond directly to estrogen or indirectly through synaptic influence with other estrogen-responsive areas. On the other hand, implantation of cannulae containing sufficiently dilute crystalline estradiol into hypothalami of ovariectomized rats revealed sites that responded to such implants with proestrous-like surges of PRL.[605]

The possibility that the implanted estradiol gained access to the systemic circulation was monitored by vaginal smears and uterine weight patterns. This study revealed that the mPOA and the ventromedial nucleus (VMN) of the hypothalamus were most sensitive to estradiol implantation, while placement in other sites involved in the estradiol-response, the SCN and anterior pituitary, did not induce proestrus-like surges of PRL.[605] Although this study suggested that the SCN is probably not estrogen-responsive directly, more recent studies have suggested that estradiol acts within the SCN ensuring the fidelity of each PRL surge. Indeed, administration of estradiol increased the expression of the mRNA for the clock gene Cry 2 within the SCN but was without affect on the expression of Cry 1 mRNA.[608]

More recent data implicate cell bodies in the locus coeruleus (presumably noradrenergic) in the elaboration of the estradiol-induced surge of PRL on proestrus.[609] Specifically, lesion of the locus coeruleus blocked both the proestrous surge and the estradiol-induced surge of PRL without influencing basal secretion. Such an observation suggests the involvement of NE in the surge of PRL.

Dopamine and Candidate Prolactin-Releasing Hormones of Hypothalamic Origin

The chemical identity of the neurotransmitters and neurohormones regulating this estrogen-dependent proestrous surge of PRL is partially known. We now appreciate the fact that though the dominant hypothalamic input regulating pituitary PRL release is inhibitory, the release of the proestrous surge of PRL probably results in a delicate balance of acute removal of inhibitory input coupled with simultaneous stimulatory input from the hypothalamus.[610] It has been shown, though not without contradiction, that dopaminergic binding sites,[611] the turnover of DA,[612] the activity of dopaminergic neurons,[613] and DA levels in portal blood[614] are depressed on the day of proestrus, corresponding to an increased secretion of PRL into peripheral plasma. DAreceptors in the anterior pituitary gland have been reported to increase[611] and decrease[615] during the afternoon of proestrus. The source of DA controlling PRL secretion is believed to be tuberoinfundibular neurons (TIDA), with cell bodies in the ARN of the hypothalamus and axons terminating upon capillary tufts in the external layer of the ME (see Chapter 12). Long portal vessels emanating from these tufts deliver DA to the anterior lobe of the pituitary gland.[616,617] There is also ample evidence to suggest that DA arrives at the anterior lobe of the pituitary gland from the posterior lobe that contains axon terminals of tuberohypophysial dopaminergic (THDA) neurons, where cell bodies are found in the rostral part of the ARN.[618,619] Finally, DA may arrive from the intermediate lobe that contains axon terminals of periventricular hypophysial dopaminergic (PHDA) neurons where cell bodies are found in the periventricular nucleus.[620–622] The short portal vessels connecting the three lobes of the pituitary gland may serve as the vascular pathway through which DA is transported to the anterior lobe from the neural and intermediate lobes. Indeed, the fact that posterior lobectomy[623] or neurointermediate lobe denervation[619] results in chronic elevation of PRL secretion implicates both THDA and PHDA neurons in the control of PRL secretion.

The issue of the control of the activity of these dopaminergic neurons on proestrus is far from settled. The content of DA in the ME and portal blood declines prior to the onset of the surge of PRL secretion on proestrus.[614–616] The proestrus surge of progesterone has been implicated in the decline in dopaminergic tone in vivo,[624] while estradiol has been implicated in the short-term decline in the in vitro activity[625] and gene expression[626] of the rate-limiting enzyme in DA synthesis, tyrosine hydroxylase. Not only does the proestrous surge of progesterone suppress the expression of tyrosine hydroxylase mRNA in the ARN,[627] but it inactivates the enzyme by inducing dephosphorylation.[628] It is quite likely that both steroids play a role in the PRL-releasing decrease in DA synthesis and neurosecretion: estradiol initiating[625] and progesterone prolonging[629] the decline in tyrosine hydroxylase gene expression on proestrus. The signal for the reactivation of the dopaminergic neurons and the resultant decline in PRL secretion at the end of proestrus are the peak levels of PRL on proestrus.[630] Indeed, immunoneutralization of circulating PRL on proestrus prevents the elevation of DA concentration[631] and activation[613] of all three populations of hypothalamic dopaminergic neurons. These "short-loop" feedback actions of heightened PRL levels are likely manifest through the activation of PRL receptors coupled to signaling via signal transducer and activator of transcription 5 (STAT5) in TIDA neurons.[632]

The induction of an estrogen-stimulated proestrus-like surge of PRL is associated with a decline of DA levels in portal blood.[633] Such evidence suggests that DA is the physiological PRL inhibitor prior to the afternoon of proestrus and that a diminution of the levels of this neurohormone in portal blood is requisite for a surge of PRL secretion during the afternoon. Based on pharmacologic approaches,[599] candidates for the converse stimulatory hormones are many. The earliest physiological evidence pointed to the tripeptide TRH as the stimulatory neurohormone on the afternoon of proestrus. Indeed, TRH is a potent stimulator of PRL release.[599] The concentration of TRH in hypophysial portal plasma increases on the afternoon of proestrus, corresponding to the times of the PRL surge.[634] However, a concomitant surge of TSH does not accompany the proestrous surge of PRL,[635] and suckling, a potent stimulus for PRL release, does not evoke release of TSH.[635] It is possible that during the estrous cycle, receptors on lactotrophs are more abundant or have a higher affinity for the peptide than receptors on thyrotrophs, thus allowing the lactotroph the most sensitive secretory response to minute changes in levels of TRH in portal plasma. Immunoneutralization of TRH during proestrus has resulted in attenuation[211] or merely altered the timing[636] of the proestrous surge of PRL. Nevertheless, it is now inferred from studies in ovariectomized, estrogen-treated rats[633] and lactating rats[637–639] as well as in vitro studies[640] that optimal PRL release on proestrus in response to estrogen and progesterone or during lactation in response to suckling requires a diminution in DA release in concert with an increase in a PRH release into portal blood.

Studies have provided renewed interest in OT actions on the proestrus pituitary gland as an important determinant of the proestrous PRL surge. Plasma concentrations of OT are increased in response to estradiol,[641] and OT concentrations in portal blood reach peak values on the afternoon of proestrus.[178] Both passive immunoneutralization of OT[180,642] and administration of an OT receptor antagonist[179] inhibit release of the proestrus PRL surge. In addition, estradiol secretions may increase the responsiveness of pituitary lactotropes to the stimulatory actions of OT.[643] Thus, lactotrophs are significantly more responsive to OT and show enhanced intracellular Ca^{++} responses when tissues are harvested on proestrus versus diestrus. These effects may be mediated by upregulation of both OT receptor levels and enhancement of downstream signaling pathways.[644] Though not as thoroughly documented, there is abundant evidence that VIP may play a physiological role as a PRH on the afternoon of proestrus (see Ref. 645 for references). On the other hand, somatostatin, GABA, atrial natriuretic hormone, vasopressin, calcitonin, NPY, galanin, substance P, bombesin-like peptides, neurotensin, and even DA itself have been implicated as PRHs, but their physiological significance has not been satisfactorily unmasked at this time (see Ref. 645 for references).

As noted in a previous section, peptides isolated from bovine hypothalamus,[646] when synthesized,[647] specifically stimulated the release of PRL from pituitary cells of lactating rats. Two peptide sequences of 20 and 31 amino acids were isolated and quickly named prolactin-releasing peptide (PrRP). The PrRP mRNA for each is most abundant in the medulla oblongata,[648–650] whereas only moderate amounts are found in the hypothalamus and the anterior lobe of the pituitary gland.[649,651,652] Neither the 20- nor 31-aa peptide will stimulate PRL secretion from pituitary cells obtained from male rats; those obtained from random cycling female rats respond only to the highest dose of either peptide.[653] When administered intravenously, the 31-aa peptide stimulated PRL secretion in male as well as female rats in a dose-dependent manner with females responding in a more sensitive manner, particularly during proestrus.[654]

Immunocytochemical approaches have revealed PrRP-positive fibers in the paraventricular and supraoptic nuclei of the hypothalamus and in the neural lobe of the pituitary gland, with cell bodies found in the DMN and VMN of the hypothalamus.[652,655] Interestingly, the presence of PrRP-positive fibers in the neural lobe and the ability of the peptide to release OT[656] suggests that PRL-releasing properties of PrRP may be mediated through OT. Alternatively, the absence of PrRP-positive fibers in the external layer of the ME and presence in the posterior lobe of the pituitary gland[652,655,657] suggest the PrRP may reach the anterior lobe through the short rather than the long portal vessels. Though meager, some studies suggest that PrRP may play a role in the preovulatory release of PRL and even LH on proestrus.[658–671] In contrast, emerging literature illustrates the role of PrRP in autonomic homeostasis.[662] Indeed, PrRP has been implicated in regulation of cardiovascular function,[663–665] stress responses,[666–669] ingestive behavior and energy balance,[670–672] and sleep.[673–675] Taken together, it is clear that the rush to (mis-) name this peptide has not led to an unambiguous conclusion as to a physiological role.

Possible Roles for Prolactin Secreted during the Estrous Cycle

Although it is quite clear that PRL is luteotrophic during pseudopregnancy and early pregnancy, an indispensable role for PRL in control of the hypothalamo–pituitary–ovarian events of the estrous cycle has not yet been firmly established. It was initially suggested that the proestrous surge of PRL plays a luteolytic role during the estrous cycle.[676,677] However, the corpora lutea are already secreting relatively small amounts of progesterone by proestrus, so it seems unlikely that such input is necessary. On the other hand, a role for the proestrous surge of PRL in control of female sexual behavior has been suggested.[678] Inhibition of the proestrous surge of PRL with a DA agonist attenuates the lordosis quotient (LQ) on proestrus, while replacement with ovine PRL restores the LQ to normal. The enhanced LQ is also associated with the estrogen-induced PRL surge in ovariectomized rats. However, it is not certain at this point if PRL has a direct physiological role in enhancing female sexual behavior or if it may act by stimulating adrenal progesterone secretion[678] (see also Chapter 50). Finally, both pituitary and ovarian roles have been attributed to PRL secreted during the estrous cycle. Specifically, the proestrous surge of PRL may attenuate the pituitary GnRH-receptor level.[679] This would explain the decline in GnRH receptor that occurs on proestrus. Secondly, within the granulosa cells of the rapidly developing ovarian follicle on proestrus, the surge of PRL may be responsible for inhibiting estrogen-synthesizing aromatase enzyme activity,[680] as reflected in the rapid decline in blood estrogen levels on the early afternoon of proestrus after the PRL surge begins.[52] However, interference with either of these activities does not appear to have a major impact on the regular expression of the normal estrous cycle in the rat.

REGULATION OF THE CORPUS LUTEUM

Rescue of the Corpus Luteum

Earlier in this chapter, it was noted that the corpora lutea formed during the estrous cycle of the rat were only ephemerally active structures. That is, except for a brief interval shortly after ovulation, the corpora lutea do not secrete sufficient progesterone to maintain a

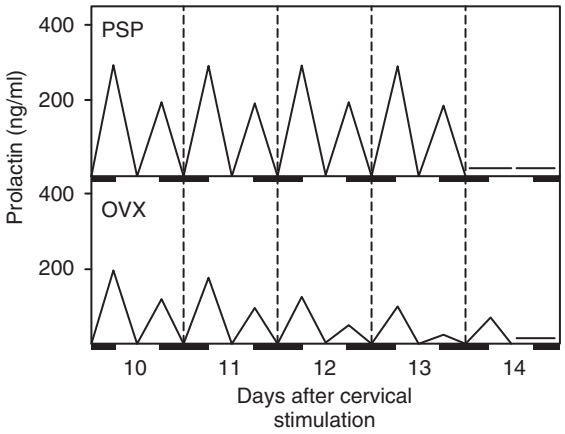

FIGURE 26.15 Diagrammatic representation of the nocturnal and diurnal surges of prolactin on days 10–14 after cervical stimulation in animals with intact ovaries (PSP, upper panels) and long-term ovariectomized rats (OVX, bottom panels). Values on the ordinate are approximate serum hormone concentrations (ng/ml NIAMDD-PRL-RP-1). Black bars on abscissa are the dark intervals in the animal quarters (18:00–06:00 h). During PSP the larger nocturnal and smaller diurnal surges persist for 12–13 days and end abruptly. The surges of prolactin in OVX rats are smaller than those of PSP rats. Moreover, as the days following cervical stimulation elapse, the magnitude of the surges gradually wanes. However, the surges themselves persist beyond a normal PSP.

decidual reaction in the uterus. Thus, the luteal phase in the rat is relatively brief, unlike that in species with spontaneous prolonged functional luteal phases of 14–16 days duration, such as guinea pigs, sheep, cows, pigs, monkeys, and humans (Chapter 23). However, the corpora lutea can be "rescued" from the quiescent state and transformed to the active state[52,681] by injection of PRL or transplantation of pituitary glands beneath the kidney capsule, which secrete copious amounts of PRL when removed from hypothalamic restraint.[682] In addition, corpora lutea may be rescued on diestrus by mating with a male rat on the previous proestrus. The corpora lutea of pregnancy persist for 20–22 days. Finally, mating with a sterile male or artificial stimulation of the uterine cervix results in a period of extended luteal function lasting 12–14 days. This interval is known as pseudopregnancy and is characterized by a period of heightened PRL secretion from the pituitary gland in response to the mating stimulus.[683,684] It is this PRL secretion that initially "rescues" the corpus luteum on diestrus[52] and allows it to respond to other luteotrophic factors.[58]

The Unique Pattern of Prolactin Secretion

Rather than being constantly elevated, the pattern of PRL secretion in response to mating is characterized by two daily surges.[683–685] One of these surges, called diurnal, begins in the afternoon (13:00–15:00 h), reaches peak values during the early evening (17:00–19:00 h), and returns to basal levels by 24:00 h. The other surge, called nocturnal, begins by 01:00 h, peaks by 03:00 to 07:00 h, and approaches baseline values by 11:00 h. This recurrent pattern (Figure 26.15) of PRL secretion continues throughout pseudopregnancy but ends abruptly by day 12 or 13.[686,687] This does not represent the typical neuroendocrine response to an acute stimulus. On the contrary, the PRL secretory response to suckling exemplifies the typical neuroendocrine reflex. Temporally, the response is tightly coupled to the stimulus. Within minutes of introduction of hungry pups, the nursing mother's blood levels of PRL increase. In addition, the magnitude of the increase is related to the intensity of the stimulus. That is, larger litters induce release of more PRL from lactating mothers than do smaller litters. The response is tightly stimulus-bound in that each subsequent secretory episode requires reinforcement by reapplication of the suckling stimulus provided by the pups. In contrast, the PRL secretory response to mating has some unique features not shared with other neuroendocrine reflexes.

Unlike the constantly elevated PRL secretion induced by suckling, mating-induced PRL secretion is surge-like, coupled to the time of day,[688] and is repetitive even without reapplication of the stimulus.[683,684] It should be noted that the requirements for mating-induced PRL secretion and the characteristics of this phenomenon are somewhat different in mice. In contrast to the induction of pseudopregnancy in rats, this process requires receipt of an ejaculate from the male.[689] Furthermore, mice exhibit a single diurnal PRL surge on day 6 of pseudopregnancy.[689] Differences in the pattern of FOS expression in the brains of pseudopregnant mice versus rats[689] suggest that the neural mechanisms that mediate the mating-induced PRL release patterns are also not identical in these two species.

Control of Mating-Induced Prolactin Secretion

Ovarian and Uterine Controls

The PRL surges in rats are somewhat independent of the ovaries in that they continue in an attenuated form if the ovaries are removed within hours of cervical stimulation or can be initiated by and continue for several days after brief cervical stimulation of long-term ovariectomized rats.[686,690] Thus, the basic neuroendocrine reflex remains in the absence of ovarian steroids. However, ovarian steroids do play a role in the control of mating-induced PRL surges. Progesterone magnifies the nocturnal surge and prolongs the recurrent nature of both the nocturnal and diurnal surges of PRL.[686,690] Moreover, estradiol magnifies the diurnal surge and abolishes the nocturnal surge. Though the steroids are not required for initiation or maintenance, they seem to play a role in the termination of these surges on a timely basis. The surges end abruptly by day 12–13 in

pseudopregnant rats (Figure 26.15) but wane gradually in ovariectomized, cervically stimulated rats through day 14.[686] This result implies an ovarian role in their abrupt termination. Indeed, the diurnal surge is terminated by the gradual withdrawal of progesterone as the corpus luteum wanes at the end of pseudopregnancy, whereas the nocturnal surge is terminated by the rising level of estradiol secreted by the newly developing follicle at this time.[686]

Just as the disappearance of PRL surges at the end of pseudopregnancy is associated with a decline in luteal activity,[686,691,692] the presence of the surges beyond day 13 is associated with lengthening of the functional lifespan of the corpus luteum. Specifically, in hysterectomized rats, regression of the corpus luteum is prevented[693] and the surges of PRL continue for several days beyond a normal pseudopregnancy.[596,694] Although it is possible that the PRL surges are terminated indirectly because of regression of the corpus luteum induced by a uterine luteolytic agent,[693] the uterus may also secrete a substance that acts directly at the hypothalamo–pituitary axis to terminate PRL surges. Indeed, the nocturnal surges of PRL are still present 16 days after cervical stimulation in long-term ovariectomized rats in the absence of the uterus but not when the uterus is in place.[694] Because there is no luteal tissue to be maintained in these animals, the continuation of the surge must be due not to persistent progesterone secretion, but to direct restraint provided by the uterus. In fact, the uterus must be present for the ovarian steroids to effectively inhibit the surges of PRL secretion.[695] This implies that the nonpregnant uterus may secrete a substance that acts at the hypothalamo–pituitary axis to modify PRL secretion.

Prolactin-inhibitory activity has been extracted from the uterus and shown to be specific for its tissue of origin and hormone on which it acts.[696] Moreover, it has been demonstrated that uterine epithelial cells secrete the activity, while its presence in peripheral blood is correlated with the presence of the uterus.[697] Although there are many questions remaining unanswered regarding the PRL inhibitory activity of the uterus, these data suggest that the nonpregnant uterus may function as an endocrine organ regulating PRL secretion.

Neural Sites Mediating the Prolactin Secretory Response to Cervical Stimulation

The pelvic nerve is the primary sensory afferent from the cervix.[698] The message provided by cervical stimulation ascends to the brainstem through the anterolateral columns of the spinal cord. The central neural areas responsive to cervical stimulation include the lateral reticular nucleus, the central gray, the gigantocellular reticular nucleus, and the dorsal raphe nucleus.[699–701] The induction of nocturnal surges of PRL can be prevented by transections in the ventromedial and lateral part of the midbrain.[702]

Within the hypothalamus, three areas have been implicated in the control of PRL release in response to cervical stimulation. Lesions of the dorsal mPOA cause repetitive pseudopregnancies[135] characterized by nocturnal but not diurnal surges of PRL.[136] Moreover, the lesion will block the diurnal surge induced by cervical stimulation.[136] These studies suggest that the mPOA possesses two types of neurons: those tonically inhibitory to nocturnal surges and those potentially stimulatory to diurnal surges. Cervical stimulation acts by inhibiting the former and stimulating the latter.[703] Because destruction of the mPOA leads to consecutive spontaneous luteal phases approximating those of other mammals, one can speculate on the evolutionary significance of the primacy of the mPOA. The activation of repetitive luteal phases by destruction of the mPOA suggests that the rat has a short estrous cycle characterized by a quiescent corpus luteum due to the tonic inhibitory activity provided by the mPOA over secretion of the luteotrophic hormone, PRL. Perhaps phylogenetically, an active mPOA that stimulates ovulation-inducing LH surges and inhibits luteal-activating PRL surges in rodents is the more primitive form. The more advanced form may be mammals, which are not dependent on the mPOA for LH secretion, ovulation, and subsequent luteal activation. Indeed, rhesus monkeys secrete perfectly normal LH surges in response to estrogen when only their medial basal hypothalami are intact[704] (see also Chapter 28).

The second area implicated in mating induced PRL secretion includes the DMN and the VMN of the hypothalamus. Electrical stimulation of the DMN-VMN induces a pseudopregnancy characterized by nocturnal and diurnal surges of PRL.[136,705] However, a lesion of this area does not interfere with estrous cyclicity, the ability to mate and support a pregnancy to term, or secrete a nocturnal surge of PRL.[705–707] Such a lesion only interferes with the diurnal surge of PRL.[706] In fact, the intact DMN-VMN is required for the stimulatory but not the inhibitory control of the PRL surges by the mPOA.[708] The third area of the hypothalamus shown to be important in mating-induced PRL secretion is the SCN. When lesioned, both surges of PRL that had been induced by mating are completely abolished.[709] Because this area has been characterized as a master pacemaker for PRL release,[710] it is probably the timer involved in transducing the time-of-day signal for these regular PRL surges.[105] At the moment, the functional interrelationships between these areas is not yet fully appreciated.

Neuroendocrine Control of the Prolactin Secretory Response to Cervical Stimulation

Although there is a reasonably expansive literature describing the neuroendocrine control of suckling-induced

PRL secretion,[599] the same is not true describing control of mating-induced PRL release (see also Chapter 12). An early study described the relationship between DA levels in hypophysial portal blood and the release of PRL surges in cervically stimulated rats.[711] As expected, there was an inverse relationship between DA levels in portal blood and PRL surges in peripheral blood. That is, during the surges, DA levels were 36% lower in cervically stimulated than control ovariectomized animals, while during intersurge intervals there was no difference between DA levels in portal blood of control and cervically stimulated rats.

The reason for this periodic inhibition of DA release into portal blood has been studied using the accumulation of L-dopa in the ME as an index of TIDA nerve activity.[712] In this assay system, the amount of L-dopa accumulated in the ME is inversely related to the activity of dopaminergic neurons. The activity of these dopaminergic neurons, in turn, is inversely correlated with the PRL surges induced by mating and may indeed be controlled by them.[712] Thus, as the PRL surge occurs in response to diminution of DA secretion into portal blood, these high levels of PRL may feedback to stimulate the activity of these TIDA dopaminergic neurons, which results in DA release into portal blood and consequent inhibition of PRL secretion.[713] Such feedback loops result in phasic rather than enhanced basal secretion and may be the basis for the two daily surges of PRL secretion in mated animals. This issue, coupled with the possible involvement of a mating-induced PRL releasing factor, is currently under study.

To determine if the decrease in DA levels is solely responsible for release of PRL in cervically stimulated rats, the synthesis of DA was blocked with α-methyl para tyrosine in ovariectomized noncervically stimulated rats. Dopamine was then infused peripherally at rates that resulted in portal blood levels simulating those of cervically stimulated rats.[711] Dopamine concentration in physiologic ranges inhibited PRL secretion, but pharmacologic quantities were required to suppress PRL to basal levels characteristic of the intersurge intervals. Moreover, a 36% decrease of infused DA resulted in only a 1.5-fold increase in PRL levels, while a 36% spontaneous decrease in DA secreted into portal blood in the cervically stimulated rat was associated with a four- to fivefold increase in PRL secretion.[711] Such observations argue for the involvement of another additional inhibiting factor or more likely a PRL releasing factor activated by cervical stimulation.

Studies have revealed the basic components of the neuroendocrine mechanisms producing the mating-induced PRL secretory rhythm. These involve reciprocal interactions between PRL, DA, and OT, with VIP input from the SCN. As we have noted, hypothalamic DA inhibits lactotroph activity via activation of D2 receptors.[714] PRL exerts feedback control over its own secretion

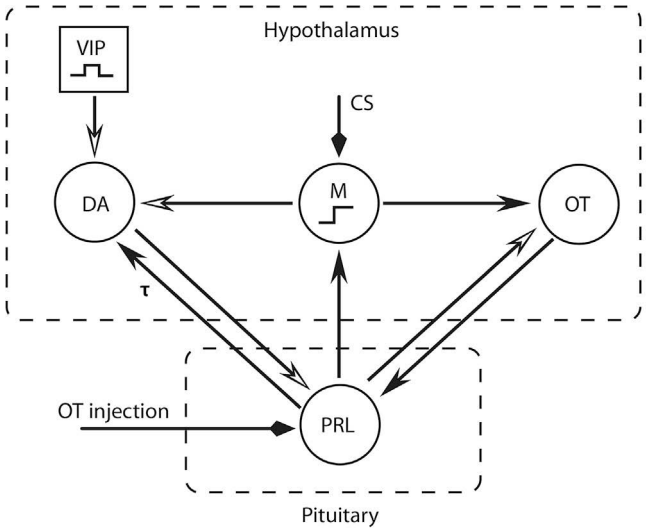

FIGURE 26.16 Proposed model for the prolactin (PRL) rhythm initiated by cervical stimulation (CS) or by oxytocin (OT) injection. Closed arrowheads indicate stimulatory actions, open arrowheads indicate inhibitory actions. The stimulation of dopamine (DA) neurons by PRL has a delay of τ hours. A pulse of vasoactive intestinal polypeptide (VIP) from suprachiasmatic neurons occurs every morning, setting the phase of the PRL oscillation. The memory, M, is switched on by CS or by PRL. *Adapted from Ref. 721.*

by activating its cognate receptors in hypothalamic DA neurons and stimulating the production and release of DA; these actions are likely mediated by phosphorylation and hence activation of tyrosine hydroxylase, as well as a more protracted increase in tyrosine hydroxylase gene expression.[715,716] A rhythm of PRL secretion would thus be predicted to occur as a consequence of the integration of these antiphasic, temporally locked feedforward and feedback actions. Additional findings have demonstrated that VIP neurons in the SCN, which innervate and inhibit dopaminergic neurons in the ARN in the morning hours,[717] appear to dictate the timing of the nocturnal PRL surge and amplify it relative to the diurnal surge.[718] The importance of this input has been confirmed by the finding that administation of VIP antisense oligonucleotides in the SCN alter PRL rhythms in cervically stimulated rats.[719] The actions of OT, functioning as a PRL-releasing factor in lactotropes, were also recently shown to be a requisite element of the PRL rhythm-generating mechanism. Administration of an OT receptor antagonist that does not penetrate the blood–brain barrier blocks the cervical stimulation-induced PRL rhythm.[720] Freeman and colleagues have thus proposed a model for the neuroendocrine mechanisms that govern the cervical stimulation-induced PRL secretory rhythm,[721] as depicted in Figure 26.16. The rhythm is mediated by reciprocal interactions between pituitary lactotropes and hypothalamic DA neurons, wherein DA suppresses PRL secretion and PRL stimulates DA release. Peaks of these two activities occur out

of phase. The rhythm is also facilitated by OT release from neurons of the paraventricular nucleus, at neurovascular junctions in the ME and posterior pituitary,[722] and its stimulation of PRL release from lactotropes.[179,642] Prolactin also exerts a rapid inhibitory feedback effect on OT neurons.[723,724] It is proposed that cervical stimulation initiates these cyclic events through induction of a "memory" or neural program that continues to operate for many days past the application of the cervical stimulus. The cellular basis for this memory is unknown, although a recent study showed that PRL itself is capable of programing the same mechanism.[725] Evidence suggests that PRL release does not mediate cervical stimulation induction of the rhythmic secretory program,[721] and thus its cellular basis remains to be clarified.

Significance of the Short Luteal Phase

As noted earlier, the corpora lutea of rats, mice, and hamsters secrete progesterone for only a brief period after ovulation. In the absence of spontaneous luteotrophic support, the waning corpora lutea marshal in new waves of ovarian follicles and hence new ovulation. Due to the brevity of the luteal phase in rodents, ovulation recurs every 4–5 days. One can speculate on the selective advantage of short cycles. In those cycles in which an ovulation is not associated with a fertile mating and conception, it would not be advantageous to have a protracted period, the luteal phase, in preparation for continuation of a pregnancy that did not take place. On the other hand, it seems most efficient if an active luteal phase that prepares the uterus for a conceptus is only associated with a mating stimulus. In that regard, a luteotrophic mechanism activated by a mating stimulus is an example of such an efficient system.

CONCLUSION

The neuroendocrine mechanisms that govern the rat estrous cycle share many essential features with neuroendocrine systems controlling ovulatory cycles in most other species. As in other animals, the tempo and sequence of events are orchestrated by hormone signals conveyed within the hypothalamic–pituitary–ovarian axis. A hypothalamic GnRH pulse-generating mechanism sustains basal gonadotropin secretions throughout the cycle, and FSH and LH secretions thereafter direct folliculogenesis and steroidogenesis, respectively. Ovarian hormones—principally estradiol, progesterone, and inhibin—exert negative feedback regulation of pulsatile GnRH release through effects on neuronal circuitries that control the GnRH pulse generator; they additionally exert direct inhibitory effects on gonadotrope synthesis and secretion, in part by modulating responsiveness to GnRH stimulation. Basal LH secretion prompts a sustained high level of estradiol release from ripening ovarian follicles at mid cycle. The increased level of estradiol then evokes a preovulatory gonadotropin surge through two integrated mechanisms—induction of an antecedent preovulatory GnRH surge, and massive priming of the pituitary gland to stimulation by the GnRH surge. An estradiol-dependent, GnRH self-priming effect constitutes part of this pituitary up-regulatory process. Ovulation is subsequently triggered by the LH surge.

Chief among the differences in rats versus other species is the tight control of the GnRH surge by the photic-entrained circadian clock. A daily neural signal specifies a temporal window on the afternoon of proestrus for the release of the preovulatory GnRH surge. A principal function of the estradiol surge is to couple the transmission of this daily neural signal to the GnRH release process. This coupling process may be mediated by ERα activation in neurons resident in the AVPv; evidence suggests these may be at least in part neurons that produce the GnRH secretagogue, kisspeptin. A direct afferent projection by kisspeptin neurons to GnRH neurons likely conveys at least a portion of this signal, resulting in KISS1R activation in GnRH neurons and release of the GnRH surge. The ensuing LH surge induces progesterone secretion on the evening of proestrus, which serves to further amplify the LH surge and to prevent its reoccurrence the next day. In parallel with the positive feedback actions of estradiol and progesterone on GnRH and LH, the sequential exposure of the hypothalamus to these hormones induces a behavioral heat that begins in the evening of proestrus. Thus, the timing of the GnRH and LH surges on the afternoon of proestrus ensures that ovulation occurs in temporal register with sexual behavior, an adaptive mechanism that greatly increases reproductive efficiency.

The secondary surge of FSH on the morning of estrous also represents a unique feature of the 4- to 5-day rodent estrous cycle. The biological role of this singular release of FSH is to recruit ovarian follicles destined for ovulation in the upcoming cycle. A drop in inhibin secretion, engendered by the primary LH surge, comprises one major signal for release of the secondary FSH surge. A role for PR activation in the gonadotrope has also been indicated in the stimulation of this secretory event.

The short luteal phase of the rat estrous cycle also stands in contrast to the protracted luteal phases in most other mammals. The recruitment of PRL as a luteotropic hormone in this species, and one that is engaged in full by mating, appears to be another adaptive mechanism that promotes reproductive efficiency in animals with short reproductive cycles. The circadian clock again appears to have been coopted as a mechanism that drives a characteristic PRL secretory mechanism that persists as a biphasic, daily pattern of release following mating.

Both similarities and differences between rats and other species will continue to invite further research. Major questions remain, for example, about the molecular and cellular basis of GnRH pulsatility and its regulation by ovarian steroids, mechanisms that appear to be common among the neuroendocrine control systems of most mammals. The cellular targets and mechanisms that engage negative feedback in the hypothalamus remain particularly unclear. Progress in understanding these phenomena will undoubtedly be made in the coming years, and will shed new light on the dysregulation of pulsatility and resistance to negative feedback that are associated with infertility syndromes in humans. The photoperiodic regulation of the proestous GnRH surge in rats reflects an inherent difference in the transmission of positive feedback signals in this species versus the sheep, monkey, and humans. A fuller understanding of the mechanisms that link the circadian clock to the GnRH release system will hopefully be gained, allowing dissection of mechanisms that impart circadian timing mechanisms from a more conserved "core" surge generating process that occurs in a wider variety of species. Future research will also hopefully clarify the molecular mechanisms that mediate the differential release of FSH and LH, a process that occurs most robustly during the morning hours of estrus. In addition to a drop in inhibin levels, the additional mechanisms by which activin, follistatin, and other pituitary factors may contribute to this process are far from resolved. Moreover, the cellular mechanisms that integrate these signals in these and other instances of singular FSH release remain to be determined. Likewise, the neural integrative mechanisms that control mating-induced PRL surges await further analysis.

Finally, I have not included in this chapter any discussion of the mechanisms that may mediate the effects of stress, illness, alterations in nutrition and/or energy balance, season, and changes in any other important neuroendocrine variables on the rat estrous cycle. As discussed in several other chapters, such studies are ongoing in numerous laboratories and continue to provide important information relative to human and animal reproductive cycles, fertility, and infertility. I hope that the body of fundamental knowledge about the neuroendocrine control of the estrous cycle, as updated and presented in this chapter, will continue to inform and guide these studies into the future.

Acknowledgments

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CHAPTER

27

Control of the Ovarian Cycle of the Sheep

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INTRODUCTION

Although significant progress has been made in some aspects of our understanding of the ovine estrous cycle, much of the information in the third edition of this volume does not need updating and remains substantially the same. We have indicated those areas where significant advances have occurred in the text, which include (1) the role of kisspeptin and neurokinin B in control of gonadotropin-releasing hormone (GnRH) secretion, (2) intraovarian mechanisms controlling follicular development and hormone secretion, (3) the regulation of follicular waves during the luteal phase, and (4) the importance of the episodic pattern of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), membrane progesterone receptors, and intracellular Ca^{++} to luteolysis. New information on mechanisms underlying seasonal breeding and pheromonal control of reproductive function discussed in more detail includes (1) a potential role for thyroid-stimulating hormone (TSH) from the pars tuberalis, and local conversion of T_4 to T_3 in mediating the actions of melatonin during the transition to anestrus; (2) identification of the components, including arcuate kisspeptin neurons, of the neural circuitry inhibiting GnRH in anestrus; and (3) determination of the olfactory pathways and the possible roles of learning and memory in the ability of males to induce ovulation in anestrous ewes. Because of space constraints, we have limited the number of references and, with the exception of classic historical citations, have generally included only more recent references; many important early references can be found in our previous chapter[1] (which can be obtained at http://www.sciencedirect.com/science/article/pii/B978012515400050049X), and we have cited it in the text where specific data from early work are particularly relevant. We have also had to rely on recent reviews more than we would like. Reviews that provide valuable coverage of major topics have been cited in the title of that section, while more focused reviews are cited in the text. We strongly encourage students to read the primary literature cited in these reviews rather than simply relying on the conclusions reached in the latter because the original work contains important experimental details, valuable data, and viewpoints not often found in reviews.

Overview

Patterns of reproductive function in the ewe are dominated by two distinct rhythms.[2] The first of these is a 16- to 17-day estrous cycle. The duration of this cycle is remarkably constant, with an estimated 95% of ewes showing estrous cycles within the normal range of 14–19 days.[3] There are some differences in cycle lengths among breeds and with age, but these differences are relatively small (~1 day).[3,4] The other major rhythm in ovine reproduction is an annual cycle of ovarian activity. In most breeds of sheep,[2–4] normal estrous cycles occur in the fall and winter (breeding season), but ovarian cycles cease in the spring and summer (anestrus). From an adaptive point of view, this limited duration of fertility ensures that lambs are born in the spring, when environmental conditions are favorable for their survival. In contrast to the constancy of estrous cycle length, seasonal reproductive patterns vary dramatically; some primitive mountain sheep have only one estrous cycle during the breeding season, while Merino ewes show estrous cycles throughout most of the year.[2] This variability appears to reflect the environment in which the breeds were developed, with breeds originating in harsher conditions having shorter breeding seasons.[4] It should be kept in mind

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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that these breed differences in seasonality reflect differences in the degree of ovarian activity during spring and summer. The presence or absence of ovulation represents a somewhat arbitrary dividing line in what is, in fact, a continuum of ovarian function. Thus, seasonal variations in fertility are evident even in ewes capable of breeding throughout the year and, in seasonal breeders, considerable ovarian activity (e.g., follicular development) occurs during anestrus.

The estrous cycle of the ewe is essentially analogous to the ovarian cycles of other spontaneously ovulating species with functional luteal phases, although the follicular phase is relatively brief compared to that of primates (see Chapter 33). It is thus not surprising that the neuroendocrine mechanisms controlling the ovine ovarian cycle are similar in many respects to those in primates and rats. However, the seasonal aspect of reproductive function represents an additional challenge for any model of these control systems. Namely, in the ewe, it must be able to account both for the events occurring within the estrous cycle and for the annual cessation and re-initiation of ovarian cycles. In addition to these theoretical issues, the occurrence of a seasonal anovulatory period provides a natural experimental model for examining the control of ovulation. Consequently, experiments examining the control of the estrous cycle and the annual reproductive cycle of sheep became intertwined over the years so that our understanding of each has developed in parallel. Nevertheless, it is useful for didactic reasons to consider these two cycles separately, and we will first consider the control of the estrous cycle. We will provide a historical perspective on the field after consideration of the current model for the ovarian cycle.

Methods of Analysis

The ovine estrous cycle results from the coordinated interaction of four tissues: brain, pituitary, ovary, and uterus. To understand the control of this cycle, we must analyze the communication among these tissues, which occurs largely via seven hormones: GnRH from the hypothalamus; luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary; estradiol, inhibin, and progesterone from the ovary; and $PGF_{2\alpha}$ from the uterus and corpora lutea. In analyzing the physiological interactions of these hormones, two factors are of paramount importance. First, for the action of a particular hormone to be important in controlling ovarian cyclicity, it must be evident at physiological concentrations of the hormone. Thus, particular emphasis will be placed on those studies that have avoided pharmacological hormone treatments or artificial manipulations with little relevance to physiological events. Second, changes in circulating concentrations of these hormones occur in a specific sequence at very predictable times during the estrous cycle. As a result, examination of the temporal relationship between two specific hormones provides a useful indication of the causal linkage between the two. Because of the importance of these two factors, this chapter will begin with a detailed description of the behavioral and hormonal patterns that occur throughout the estrous cycle. We then dissect the cycle into its functional components and consider the control of each component individually. The third step will be synthesis of these components into a model that can account for the events of the estrous cycle. Finally, we will briefly consider how these mechanisms are modified by photoperiod and pheromonal input to produce seasonal alterations in reproductive function.

DESCRIPTION OF THE OVINE ESTROUS CYCLE

The 16–17-day estrous cycle can be divided into a 2–3-day follicular phase and a 13–14-day luteal phase. The demarcation between a follicular and subsequent luteal phase is ovulation, whereas there is a more prolonged transition between a luteal phase and the next follicular phase because luteolysis is a gradual process. Because this is a cycle, one can theoretically start at any point in its description. However, because estrous behavior is the only outward manifestation of the ovine ovarian cycle (and can be easily monitored using vasectomized rams with marking devices), the onset of estrus is used to time the events of the cycle and, by convention, the first day of estrus is commonly designated day 0 of the ovarian cycle.

Estrous Behavior[5]

Traditionally, estrous behavior is divided into three distinct activities: attractivity, proceptivity, and receptivity. Attractivity consists of passive characteristics and behaviors that make a ewe attractive to rams, proceptivity involves activities in which the ewe is actively seeking a mate, and receptivity consists of a ewe allowing the ram to mount.[5,6] While attractivity can be identified under experimental conditions,[7] it is difficult to distinguish from proceptivity under natural conditions, so it is usually subsumed under the latter. Proceptivity is evident as an increase in motor activity at the start of estrus and can be accompanied by tail fanning and the ewe looking over her shoulder at the ram as he approaches and nudges her flank and/or anogenital region. Receptivity, which is the most common index used to monitor estrous behavior, consists of simply standing still during copulation. Copulation is fairly brief, but several copulations usually occur during a single estrous period. In general, behavioral estrus lasts 24–36 h in the ewe, but its

duration is reduced by continuous contact with males.[5,6] The start of estrus is coupled with the preovulatory LH surge so that ovulation occurs, on average, 24–30 h after the onset of estrous behavior.[3,8]

Hypothalamo–Hypophysial Unit

LH and GnRH Patterns

The ability to monitor GnRH secretion in the hypophysial portal blood of unanesthetized sheep has produced a wealth of information on the normal pattern of endogenous GnRH secretion and its control that is not available in any other species. These data demonstrated a very close correlation between GnRH and LH secretion, so we have chosen to describe secretion of these two hormones in tandem. Historically, two functionally distinct modes of LH secretion were identified in the ewe, as in other mammals (see Chapter 33). These two modes of LH, and parallel secretion of GnRH, are regulated by different feedback mechanisms and control different aspects of ovarian function. Tonic LH secretion is controlled by the negative-feedback actions of ovarian hormones, occurs in a pulsatile pattern at a low level throughout the cycle, and is important for ovarian steroidogenesis; the LH surge is triggered by high circulating concentrations of estradiol late in the follicular phase and induces ovulation and formation of the corpus luteum. As discussed in this chapter, the negative and positive feedback actions of ovarian hormones occur at both the hypothalamic and hypophysial portions of this unit.

Tonic LH secretion during the cycle (Figure 27.1) is inversely correlated to luteal function so that LH concentrations decline from days 1–9 as progesterone secretion from the corpus luteum increases,[13] remain low until day 14, and then increase during the follicular phase following luteolysis.[13–15] In contrast, when averaged over several hours, tonic GnRH secretion does not vary markedly throughout the cycle (Figure 27.1),[9,16] but these means mask a marked variation in the pattern of pulsatile GnRH release (Figure 27.2). During the midluteal phase, low-frequency, high-amplitude GnRH pulses are observed; following luteolysis, the frequency increases and the amplitude decreases.[9,16] Because GnRH pulses drive LH pulses, LH pulse frequency follows the same pattern (Figure 27.2): it is high in the early part of the cycle, decreases to a nadir late in the luteal phase, and then increases progressively throughout the follicular phase. LH pulse amplitude either does not differ between the midluteal and follicular phases or decreases during the follicular phase, so mean LH concentrations correlate with LH pulse frequencies, not amplitudes, during the estrous cycle.

The LH surge represents a brief, massive outpouring of LH from the pituitary (Figure 27.1). LH concentrations rise rapidly within 4–8 h to a peak that is 50–100 times basal levels, and then decline just as rapidly so that the surge lasts approximately 12 h. The onset of the LH surge is usually coupled to the onset of estrous behavior,[5] and the interval between the LH surge and ovulation (22–26 h) is remarkably constant. At the same time, there is a dramatic 20- to 40-fold increase in the GnRH secretion rate (Figure 27.1),[9] which reflects a change in the pattern of GnRH release from episodic to continuous.[17] This change occurs gradually, starting with increased GnRH secretion between pulses, followed by an increase in both interpulse GnRH secretion and GnRH pulse amplitude, and then continuous release during peak secretion.[17,18] Although the initial phases of the preovulatory surges of GnRH and LH are relatively synchronous, elevated GnRH secretion is maintained for many hours after the termination of the LH surge (Figure 27.1).[9]

FSH Secretion

As illustrated in Figure 27.1, FSH concentrations decline during the follicular phase to a nadir on the day before the preovulatory LH surge.[10,11,19] There is an FSH surge coincident with the LH surge, and FSH concentrations fall in parallel with LH to a nadir at 8–12 h after the preovulatory peak. FSH then increases to a second peak 20–28 h after the first, followed by a second nadir between 36 and 48 h. A gradual increase in FSH concentrations is often observed sometime between days 4 and 6 of the luteal phase,[11,19,20] which correlates with the second "wave" of follicular development (discussed later in this chapter). Small increments in FSH, lasting about a day, occur at 3- to 6-day intervals throughout the luteal phase, each preceding a wave of follicular development.[20,21]

Ovary

Follicular Dynamics[22]

Because of space limitations and the availability of an excellent review on all aspects of folliculogenesis in ruminants,[22] we have chosen to rely primarily on that publication with only selected references to the primary literature. Formation of primordial follicles begins in the ovigerous cords near the interface of the cortex and medulla of the fetal ovary around day 75 of gestation,[23] coincident with maximal germ cell numbers in the fetal ovary. Initially, each primordial follicle consists of an oocyte, surrounded by a single layer of granulosal cells; the number of these per follicle is highly variable, but correlates with the diameter of the oocyte. Granulosal cells come from pregranulosal cells that arise primarily in the surface epithelium of the developing ovary and segregate the oocytes within the ovigerous cords before these cords regress.[23] More than 75% of the germ cells are lost by day 90 of gestation, presumably by apoptosis. The ovaries of young ewes contain from 40,000 to 300,000 primordial follicles. Numbers of antral follicles

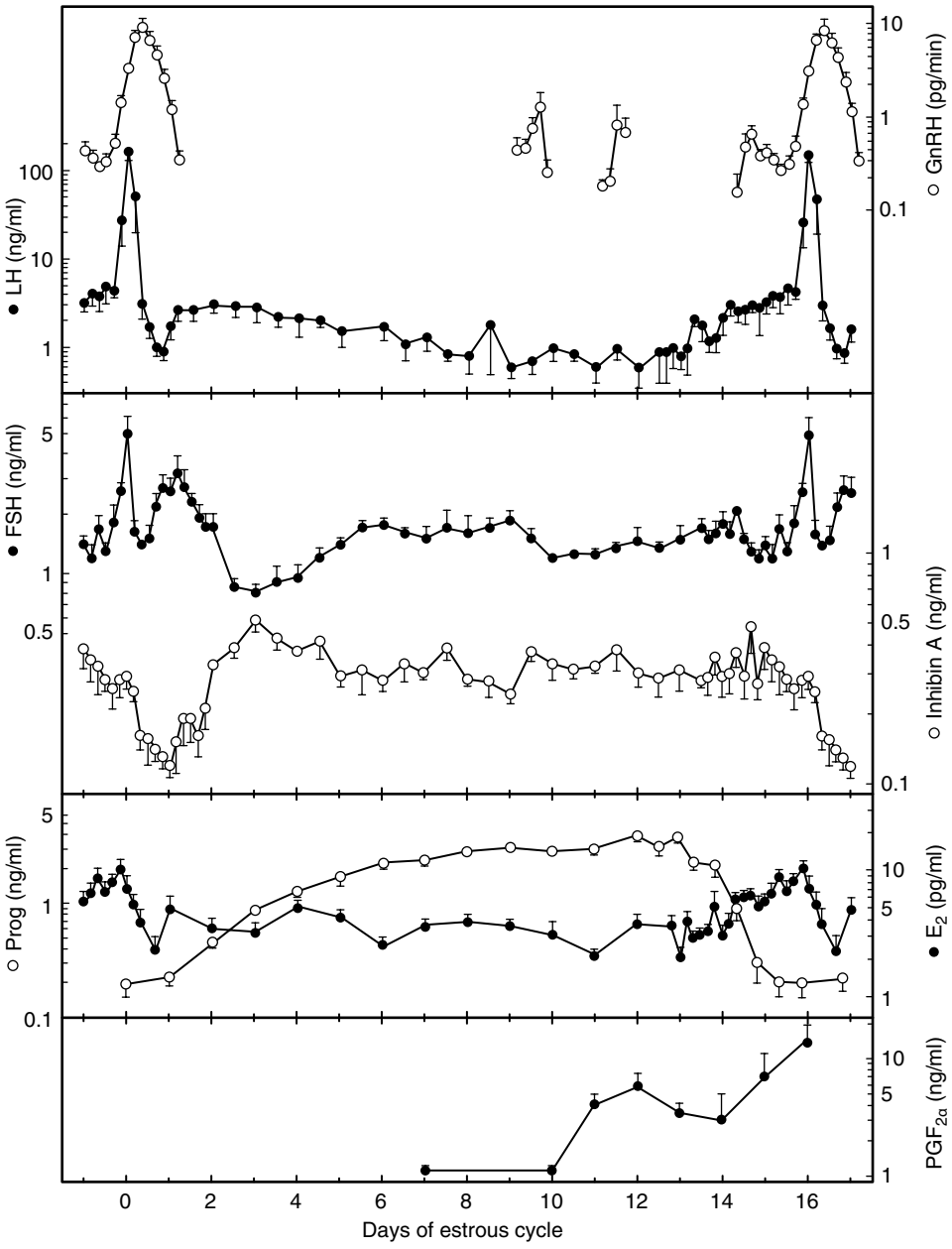

FIGURE 27.1 Hormonal patterns during the ovine estrous cycle. Changes in mean (± standard error of the mean) secretion rate of GnRH into hypophysial portal blood, peripheral concentrations of gonadotropins and major ovarian hormones, and uterine vein concentrations of $PGF_{2\alpha}$ throughout the ovine estrous cycle. Data for all hormones (except $PGF_{2\alpha}$) during the periovulatory period (days −1 to +2 and 13–17) are normalized to the peak of the LH surge (day 0 and day 16) and represent values monitored every 4 h. During the remainder of the cycle, data collected every 12 or 24 h are presented. By convention, the day of estrous behavior is designated Day 0; this is also the day of ovulation and thus the start of the luteal phase. The follicular phase begins with the first sustained fall in progesterone concentration (on Day 14 in this figure), but the transition between luteal and follicular phases occurs over the next 24 h as progestrone concentrations fall to a minimum. *Source: GnRH values were calculated from data in Moenter et al.,[9] and LH and ovarian steroid concentrations are from Goodman et al.[10] (Copyright 1981, The Endocrine Society.) FSH and inhibin A from Knight et al.,[11] and $PGF_{2\alpha}$ values are derived from data in Inskeep and Murdoch.[12]*

in ewe lambs are high at birth and fluctuate randomly during the prepubertal period, with no obvious follicular waves or correlation with FSH concentrations.[24]

Whether follicular waves, similar to those in cows, occur in adult ewes has been an ongoing controversy. In an analysis of 15 laparoscopic and ultrasonographic studies, it was concluded that waves can be demonstrated if one defines a wave on the basis of follicles that arise at 3 mm in diameter and grow to at least 5 mm in diameter (Figure 27.3).[25] However, numbers of waves ranged from two to six among studies, waves sometimes overlapped, and ovulatory follicles arose from both the penultimate and ultimate waves[26] and as early as day 9 or as late as day 14 of the cycle.[27] Interestingly, cycle length did not vary between cycles with three or four waves,[28] in contrast to cattle, in which cycles with three waves were longer than those with two waves. Detailed hormonal analyses indicated that each follicular wave was preceded by a transitory increase in FSH concentrations[1,20,21,26] and that recruitment into the growing cohort required the presence of some LH. Follicular waves continued during anestrus and were often,[29] but not always,[30] associated with increments in FSH; as in the luteal phase, the presence of some LH appeared to be essential for recruitment by FSH.[29]

Follicular selection or dominance[31] has been difficult to demonstrate in ewes, although it is supported by observations during the follicular phase.[25,32] It has been

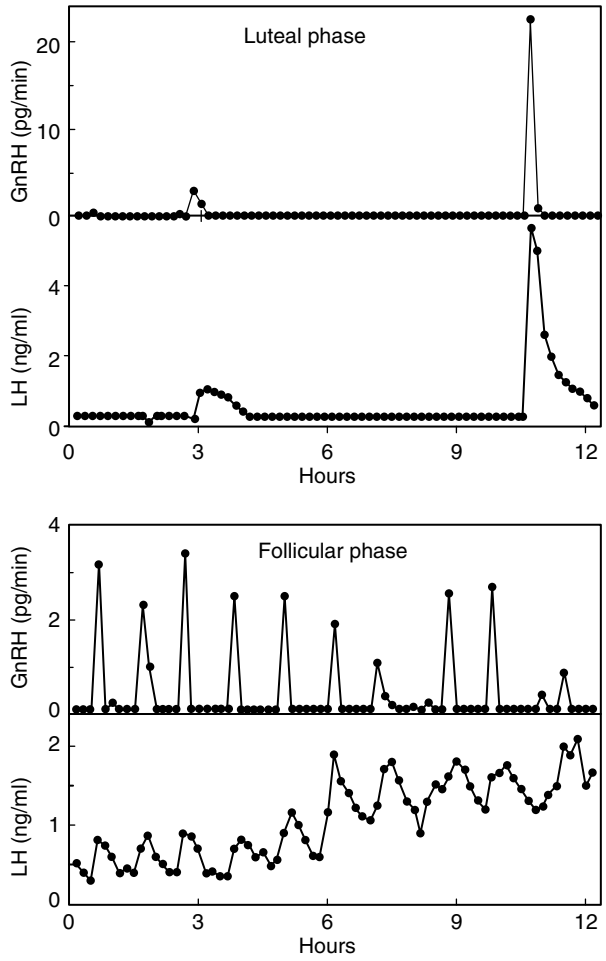

FIGURE 27.2 Episodic GnRH and LH secretion varies with stage of estrous cycle. Pulsatile patterns of hypophysial portal GnRH secretion and jugular concentrations of LH during the late luteal phase (days 12–13) and late-follicular phase (39–51 h after progesterone withdrawal). Note the difference in the scale of the *y*-axis between the luteal and follicular phases. *Source: Drawn from data provided by F. J. Karsch from work described in Moenter et al.*[9]

suggested that "potentially ovulatory" is a better term than "selected" or "dominant" for the most advanced class of follicles,[22] and the concept of follicular dominance in the ewe has recently been questioned.[33] It is thus not surprising that in many breeds of sheep, twinning occurs in a significant percentage of animals. In ewes with twins, ovulatory follicles arose over a greater range of days than in ewes with single ovulations, and twin ovulations appeared to result from reduced atresia of both smaller and larger follicles.[34] That is consistent with ovulations from both the ultimate and penultimate waves; that is, larger follicles from the penultimate wave were retained and smaller ones in the ultimate wave grew to ovulatory size and competence. More follicles from the penultimate wave ovulated in breeds of ewes with lower progesterone and more frequent pulses of LH.[26] The most rapid apparent turnover of follicles was reported in the Merino del Pais, a monovulatory breed in Spain, in which seven different follicles became the largest during an estrous cycle.[32] A subsequent analysis in three other Spanish breeds concluded that patterns of follicular turnover were not affected by breed.[35] Readers are referred to the review by Scaramuzzi et al.[22] for models that illustrate the processes of recruitment and selection of follicles in relation to patterns of FSH and LH secretion.

Ovulation (Follicular Rupture)[36,37]

The process of ovulation in the ewe does not differ particularly from that described in other species, but work in ewes has contributed to greater understanding of the role of the ovarian surface epithelium and of the involvement of tumor necrosis factor alpha (TNFα) in rupture of the follicle. Therefore, we will focus this description on these events; a more detailed description of this process can be found in Chapter 22. Key steps in the ovulatory cascade in ovine follicles (Figure 27.4) begin with an initial effect of the LH surge to increase 3′5′ cyclic adenosine monophosphate (cAMP), which blocks synthesis of estradiol from progesterone via androgens. The resulting increase in progesterone in the follicular tissue contributes to increases in secretion of PGE_2 and $PGF_{2\alpha}$, which contribute sequentially to hyperemia of the follicle and proteolytic activity (PGE_2) and to contractions of smooth muscle–type cells in the follicular wall ($PGF_{2\alpha}$). LH stimulates secretion of urokinase-type plasminogen activator (uPA) from the ovarian surface epithelium. The uPA stimulates conversion of interstitial plasminogen to plasmin, which activates latent collagenases in the apical follicular wall and releases TNFα from the thecal endothelium. Collagenolysis, potentiated by increases in transcription of matrix metalloproteinases (MMPs) in response to TNFα, and further supported by progesterone and prostaglandins, weakens and thins the apical follicular wall, leading to stigma formation and rupture. Tissue inhibitors of MMPs limit the degree of proteolytic damage and contribute to collagen remodeling during the folliculo–luteal transition.

Ovarian Hormone Secretion

The primary steroids secreted by the ovary are progesterone, estradiol, and androstenedione. Although circulating androstenedione does not appear to play a physiological role in ewes,[1] immunization against it has been used successfully to increase the ovulation rate.[38] Progesterone is secreted into the blood exclusively by the corpus luteum, whereas estradiol is secreted only by follicles.[1] Progesterone concentrations are virtually undetectable early in the cycle and then rise gradually, in parallel with increases in the volume of luteal tissue, determined using ultrasound,[39] from days 2 to 8 to reach a maximal concentration ranging from 1.5 to 4 ng/ml, depending on the breed.[1,13] Progesterone

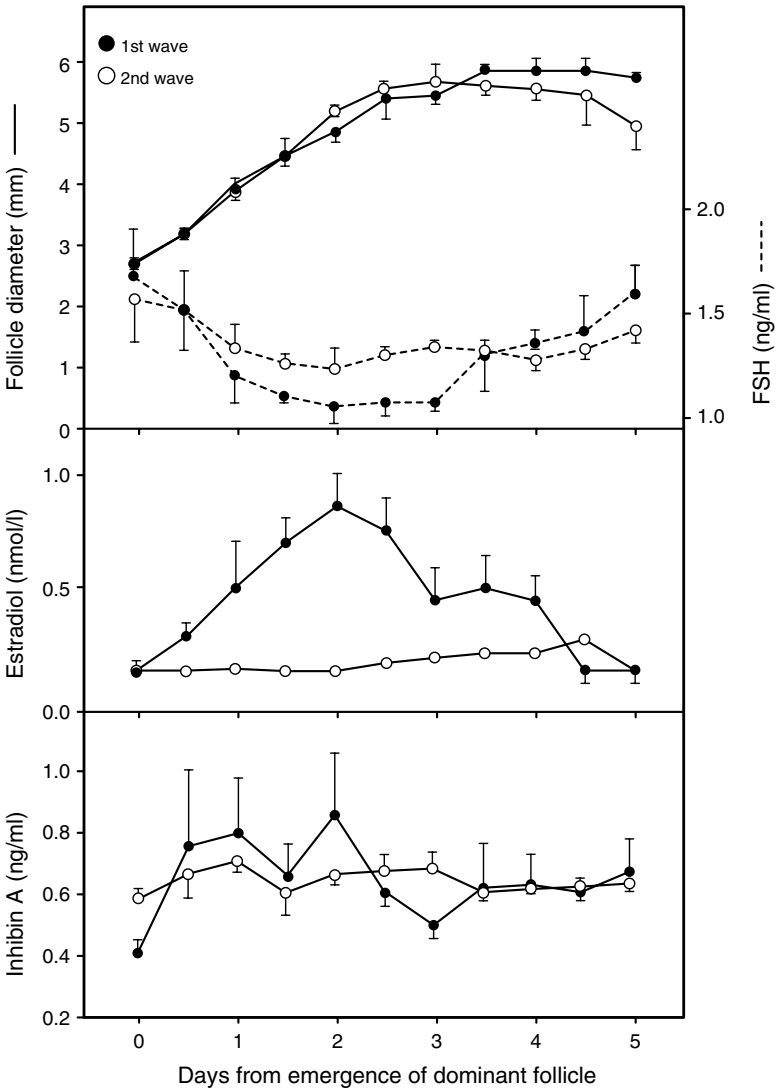

FIGURE 27.3 Follicular waves and hormonal patterns early in the luteal phase. Relationships among the diameter of the largest follicle (*top panel*, solid lines), peripheral FSH concentrations (*top panel*, dashed lines), and concentrations of estradiol (*middle panel*) and inhibin A (*lower panel*) in ovarian venous plasma during the first and second follicular waves of the estrous cycle. Values (mean ± standard error of the mean) are normalized to the day of first emergence (diameter >2.5 mm) of the largest follicle, for waves 1 (from days 1 to 4 of the cycle) and 2 (from days 5 to 9 of the cycle). *Source: Data replotted from Souza et al.[20]; reproduced by permission of the Society for Endocrinology.*

concentrations remain relatively constant from days 8 to 14, and then rapidly fall to undetectable levels over the next 1–2 days (Figure 27.1). In contrast to the rat and primate (see Chapters 26 and 28), peripheral progesterone concentration does not increase in response to the preovulatory LH surge in the sheep,[40] but it does increase within the preovulatory follicles and plays an important role in the ovulatory process (see Figure 27.4). This pattern of progesterone concentrations is remarkably constant across breeds, but there are modest breed differences in maximum progesterone concentrations. Surprisingly, breeds with high ovulation rates do not always have higher progesterone concentrations than those with low ovulation rates,[39] indicating that less progesterone is produced per corpus luteum in the former.

The principal estradiol rise occurs during the 2–3-day follicular phase of the estrous cycle (Figure 27.1): estradiol secretion begins to rise after the first drop in progesterone concentrations at luteolysis[40] and increases five- to 10-fold over the next few days.[13,14] This estradiol increment peaks at the start of the preovulatory LH surge (Figure 27.1) and then rapidly declines to basal concentrations. Estradiol secretion during this period appears to come largely from the follicle destined to ovulate, which is growing rapidly at this time. In most studies, a second peak in estradiol secretion was observed around day 4 of the cycle that correlated with the first follicular wave (Figure 27.3)[13,20,40]; mean estradiol concentrations remained relatively low during the rest of the luteal phase and showed no consistent pattern during this period (Figures 27.1 and 27.3). When measured at frequent intervals in individual sheep, both progesterone and estradiol show marked fluctuations, indicative of an intermittent secretory pattern analogous to tonic GnRH and LH secretion.[1] The episodic secretion of estradiol, but not progesterone, is temporally correlated with preceding LH pulses.

Inhibin is produced by follicles, but not the corpus luteum, in sheep,[41] and it comes primarily from granulosal cells, although low levels of mRNA for both the

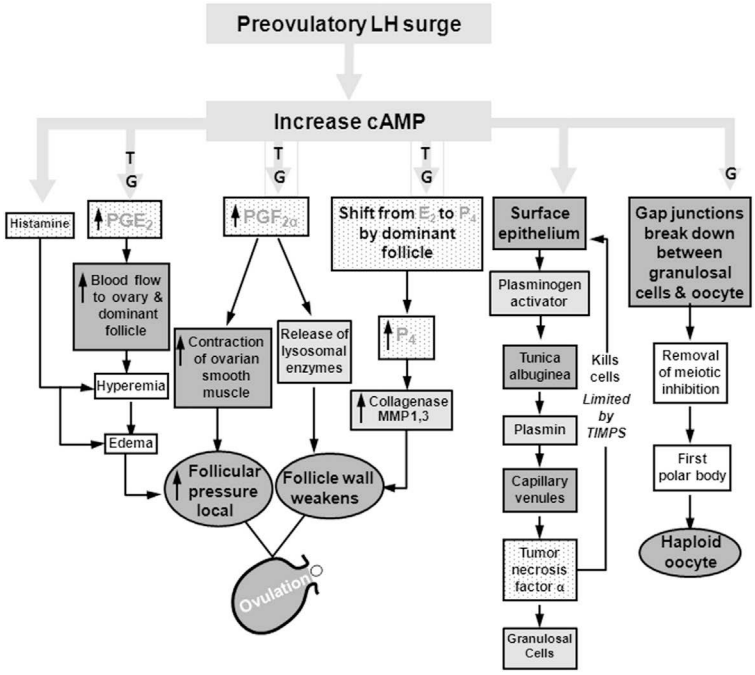

FIGURE 27.4 Sequence and sites of the process of follicular rupture and ovulation in the ewe in response to the preovulatory surge of luteinizing hormone. Hormones and cytokines are in light gray print in stippled boxes, tissues are in dark gray boxes, enzymes are in light gray boxes, and results are in dark gray ovals or structures. Hormonal and enzymatic changes in granulosal (G) and thecal (T) cells are indicated on arrows. *Source: Adapted from Murdoch et al.[36] and information provided by E. K. Inskeep from work described in Senger PL Pathways to Pregnancy and Parturition, 3rd Ed., Current Conceptions Inc., Redmon, OR; 2012: 173.*

α and β_A subunits have recently been detected in thecal cells using quantitative reverse-transcriptase (qRT)–polymerase chain reaction (PCR).[42] Although mRNA for both β_A and β_B can be found in ovine follicles, inhibin A appears to be the only form of inhibin secreted by the ovary,[11] and inhibin B could not be detected in ovine plasma or follicular fluid.[22] Large follicles are an important source of inhibin, but smaller follicles also appear to contribute significantly to ovarian inhibin secretion.[41] Because the latter are not an important source of estradiol, the ovarian secretion of these two follicular hormones is not always coupled.[11,19,41]

The development of two-site enzyme-linked immunosorbent assays was critical for accurate estimates of circulating inhibin and provided relatively consistent estimates of inhibin A secretion throughout the ovine estrous cycle (Figure 27.1). In contrast to estradiol secretion, there is no change in either peripheral inhibin concentrations[19] or inhibin secretion rate from the ovary[11] during the follicular phase. Inhibin begins to decline within a few hours after the peak of the LH surge, reaching a nadir about 24 h later that coincides with the peak of the second FSH surge. There is then an increase in inhibin secretion between days 1 and 3 of the luteal phase, followed by a slight decline over the next 1–2 days.[11,19] No further consistent changes in inhibin are seen throughout the rest of the luteal phase, although transitory decreases sometimes correlate with increases in FSH secretion and initiation of follicular waves (Figure 27.3). At this time, there is little evidence that inhibin is secreted episodically and, in any case, episodic fluctuations in secretion would be damped by the 50 min long half-life of this hormone.

Uterine Prostaglandins

Assessment of uterine $PGF_{2\alpha}$ secretion in vivo is complicated by two factors. First, it must be measured in the utero-ovarian or uterine vein because prostaglandins are metabolized almost completely in one passage through the lungs and because some metabolism occurs locally in the uterus and/or ovary.[43] Second, $PGF_{2\alpha}$ is secreted episodically,[12,40] so that blood samples must be collected relatively frequently to determine the actual secretory pattern. When these technical issues are taken into account, a consistent picture of $PGF_{2\alpha}$ secretion emerges. From days 2 to 10 of the estrous cycle, there is little secretion of $PGF_{2\alpha}$ from the uterus.[40,44] Episodes of $PGF_{2\alpha}$ secretion first appear between days 11 and 13 of the cycle, and the frequency of these episodes then increases over the next 2–3 days.[12,44] These changes in episodic prostaglandin release are reflected in mean concentrations of $PGF_{2\alpha}$ in the uterine vein, which increase late in the luteal phase, plateau during the early follicular phase, and then increase again to a peak after progesterone declines (Figure 27.1).[12,40,44]

ANALYSIS OF THE OVINE ESTROUS CYCLE

In this section, we will consider the hormonal control of each component of the ovine estrous cycle. This is not intended to be a complete review of each component, but instead will focus on those aspects of control directly relevant to events of the estrous cycle and recent mechanistic observations.

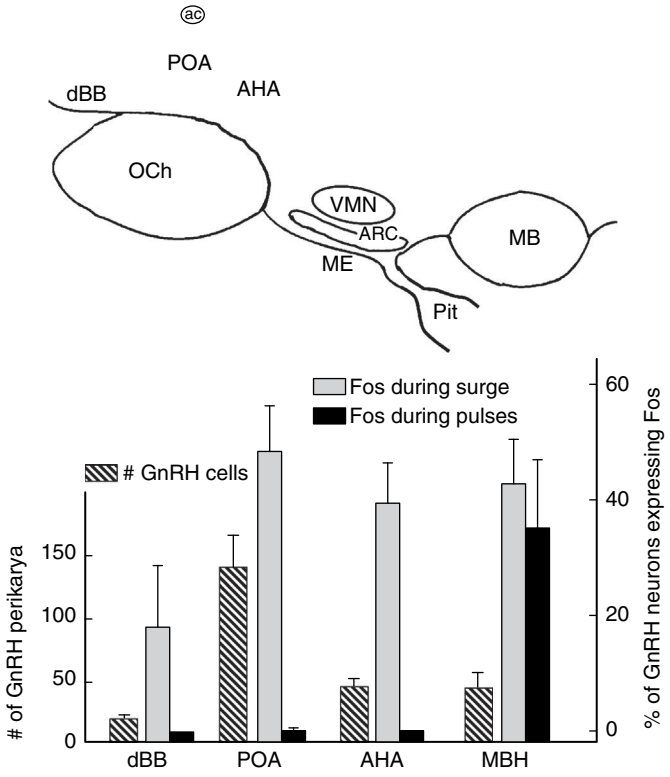

FIGURE 27.5 Diencephalic areas important for reproductive function in the ewe. *Top panel*: Schematic drawing of a parasagittal section through the ovine preoptic area and hypothalamus illustrating the major areas involved in the regulation of estrous behavior and GnRH secretion. ac: anterior commissure; AHA: anterior hypothalamic area; ARC: arcuate nucleus; dBB: diagonal band of Broca; MB: mammillary body; ME: median eminence; OCh: optic chiasm; POA: preoptic area; Pit: pituitary; VMN: ventromedial nucleus. *Bottom panel*: Distribution of GnRH perikarya (striped bars) and GnRH neurons activated (percentage containing Fos) during the preovulatory GnRH surge (gray bars) and during pulsatile GnRH secretion (black bars). Data are mean ± standard error of the mean; note that the scale for percentage of GnRH neurons expressing Fos is on the right *y*-axis. *Source: Values for GnRH perikarya and Fos expression during pulsatile secretion (Copyright 1999: The Endocrine Society) are replotted from Boukhliq et al.[46]; those for Fos expression during the surge plotted are from data presented in Moenter et al.[47]*

Control of Hypothalamo–Hypophysial Function

Neuroanatomy

As in many other species (see Chapter 11), GnRH neurons are scattered throughout the ovine diencephalon (Figure 27.5),[45] with about 50% of them located in the preoptic area (POA) and the rest found in the diagonal band of Broca (dBB), anterior hypothalamic area (AHA), and mediobasal hypothalamus (MBH), which extends from the posterior border of the optic chiasm to the rostral edge of the mammillary body and includes the arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), and ventromedial nucleus (VMN). Most of these cells are multipolar with extensive dendritic processes, but some bipolar cells can be seen. Interestingly, in contrast to nearby non-GnRH cells, the soma and dendrites of ovine GnRH neurons are almost entirely ensheathed by astroglia[48]; over 90% of the cell membrane is in direct apposition with astroglia processes in both breeding and anestrous seasons.[49] Although there is good evidence for anatomical and functional subpopulations of GnRH neurons (discussed below), there are no regional differences in the percentage of GnRH neurons projecting to the median eminence.[50] Thus, GnRH fibers arising from cells in the dBB, POA, and AHA, which travel either periventricularly or laterally at the base of the brain, are responsible for most of the GnRH terminals in the external zone of the median eminence.

The first obvious step in identifying mechanisms by which steroids act within the hypothalamo–hypophysial unit is to determine the location of steroid receptors. Thus, one can infer from the presence of receptors for ovarian steroids in gonadotropes[51] that these steroids exert feedback actions at the level of the pituitary, and, as discussed later, there is also direct evidence for this conclusion. In contrast, GnRH neurons do not contain estrogen receptor alpha (ERα)[52,53] or progesterone receptors (PRs),[54] but about 50% of them contain ERβ.[55] This dearth of steroid receptors, and the lack of evidence that ERβ is important for the control of GnRH, have led investigators to search for neuronal populations likely to mediate the feedback actions of gonadal steroids. Work over the last decade has added four new candidates to the pantheon of neurotransmitters implicated in the control of GnRH in the ewe. Thus kisspeptin,[56] galanin,[57] orphanin-FQ (OFQ),[58] and RF-amide related peptide 3 (RFRP3; sometimes called gonadotropin inhibitory hormone)[59] join the four classical neurotransmitters (dopamine (DA),[52,60] gamma-aminobutyric acid (GABA),[53] glutamate,[61] and norepinephrine[62,63] (NE)) and five neuropeptides (dynorphin,[64] β-endorphin,[52] neurokinin B (NKB),[65] neuropeptide Y (NPY),[60] and somatostatin[66]) previously identified (Table 27.1). Also of considerable interest was the report that three of these peptides (kisspeptin, NKB, and dynorphin) are co-localized in the same population of ARC neurons.[77] Because this combination is unique to the ARC (kisspeptin and dynorphin neurons elsewhere in the hypothalamus do not contain either of the other two peptides), the ARC population is now referred to as KNDy neurons.[73] Each of these 13 transmitters has been identified in boutons in close contact with GnRH neurons in the POA using light microscopy,[49,57,58,65,67,69,75,76,78] and synaptic contacts containing three of them (dynorphin, β-endorphin, and NPY) have been identified with electron microscopy.[1,69] Some of these signaling molecules are found in the same synaptic terminals; in addition to the KNDy peptides, there is a 50–60% overlap of NPY and NE,[67] and glutamate is found in 15% of the NE terminals.[61] It should be kept in

TABLE 27.1 Neurons Implicated in the Control of GnRH in Ewes

Neurotransmitter or Neuropeptide	% POA GnRH Cells Contacted	Area of Cell Bodies	% Cells Containing ERα	Afferents to POA	Effect on GnRH Release
Dopamine	12–18%	POA/AHA (A14)	3–17%[a,52,60]	No	Inhibit and stimulate
		ARC (A12)	3–13%[a,b,52,60]	No	Inhibit and stimulate
	0%[68]	RCh (A15)	0%[52]	No	Inhibit in anestrus
Dynorphin		POA/Aha	90%[b,64]		Inhibit
β-endorphin	65%[69]	ARC	20%[b,52]	Yes	Inhibit
GABA	20%[67]	POA	44%[53]		Inhibit
Galanin	Most[57]	POA	50–75		
		BNST	10–25%[c,70]		
		ARC	0%[70]		
Glutamate	73%[67]	bNST	35–70%[c,61]	No	Stimulate
		POA	41–50%[c,61]	No	
		ARC	48–77%[c,61]	Yes	
Kisspeptin	25%[71]	POA	50%[72]	Yes	Stimulate
KNDy					
Dynorphin	40%[71]	ARC	>95%[b,73]	Yes	Inhibit
Kisspeptin	40%[71]	ARC	>95%[b,73]	Yes	Stimulate
Neurokinin B	40%[71]	ARC	>95%[b,73]	Yes	Stimulate
Norepinephrine	19%[67]	Brain stem A1	20–83%[a,c,62,63]	Yes	Stimulate and inhibit
		Brain stem A2	16–60%[a,c,62,63]	No	Stimulate and inhibit
Neuropeptide Y	25%[67]	ARC	4–12%[c,60]	Yes	Inhibit and stimulate
		Brain stem[d]		Yes	
Orphanin-FQ		POA	0%[74]		Inhibit
		AHA, ARC	72%, 95%[b,74]		
RF-amide related peptide-3	20%[75]	DMH/VMN		Yes	
Somatostatin	80%[76]	VMN, ARC	29%, 13%[66]		Inhibit

[a] *Variability based on different reports.*
[b] *Also contains PR.*
[c] *Variability based on rostral–caudal differences with area.*
[d] *Based on co-localization with dopamine-β-hydroxylase in boutons contacting GnRH perikarya.*

mind that ERα-containing neurons in the ARC (but not elsewhere) also project to the median eminence,[79] as do KNDy neurons,[80] where they may contact GnRH terminals; DA synapses on GnRH terminals in the median eminence have been identified with electron microscopy.[1]

All but one of these transmitters (RFRP3) have been co-localized with either ERα and/or PR in neuronal cell bodies in the hypothalamus or, in the case of NE, in A1 and A2 groups in the brain stem (Table 27.1). These steroid-responsive populations are likely candidates for mediating the feedback actions of estradiol or progesterone, but there is little evidence on which of them project to GnRH neurons. Co-localization of KNDy peptides in boutons contacting GnRH neurons has been used to demonstrate that this population projects to one-third of GnRH neurons in the POA and about 40% of those in the MBH,[71] but this approach is not available for other potential afferents. Retrograde tract-tracing studies have demonstrated projections to the POA from ERα-containing neurons in the ARC and VMN[81]; from ARC neurons containing glutamate,[61] dynorphin, β-endorphin, DA, and NPY[82,83]; and from A1 NE neurons,[84,85] but have not demonstrated that these projections contact GnRH neurons. Anterograde tract-tracing studies have produced conflicting results, with one finding projections to GnRH neurons from the VMN[86] while the other did not.[87] The

latter study also found no input from the ARC,[87] which is not consistent with recent data using KNDy peptides as markers (as discussed above). This difference may reflect the location of the tract tracer injection, which appeared to be in the anterior portion of the ARC where there are few KNDy neurons. It should also be kept in mind that steroid-responsive neurons can influence GnRH secretion indirectly via interneurons, as has been recently suggested for A1 and A2 NE cell groups,[88] and that other neurotransmitters may well contribute to steroid negative feedback. For example, nitric oxide synthase (NOS) is co-localized with PR-containing neurons in the ARC and VMN,[89] but NOS-positive synaptic contacts on GnRH neurons have yet to be identified.

Most of the work in this field over the last decade has focused on kisspeptin, although there is now considerable new information on galanin and OFQ as well. Kisspeptin neurons in the sheep, as in other species (see Chapter 11), are found in the ARC and POA. Essentially, all of the ARC population, which also express NKB and dynorphin, contain ERα[72] and PR,[64] while about 50% of the POA kisspeptin neurons contain ERα.[72] Galanin and OFQ share several characteristics in common: (1) they are both found in all GnRH neurons (although antigen retrieval is needed to visualize galanin)[57,58,90]; (2) another population of neurons containing each peptide, but not GnRH, are distributed throughout the hypothalamus (e.g., in the PVN, AHA, and anterior portions of the ARC), and some of these contain ERα (OFQ and galanin)[70,74] and PR (OFQ)[74]; and (3) they both co-localize with known neuropeptides in the ARC (galanin with DA,[91] and OFQ with β-endorphin[74]). Galanin can act via Gal-R1, Gal-R2, and Gal-R3, and mRNA for all three are found in the POA, PVN, and ARC and increase during the luteal phase[92]; Gal-R1 protein is found in ovine GnRH neurons (5–20%, with the highest percentage in the luteal phase),[93] but Gal-R2 protein is not.[94] OFQ is considered one of the endogenous opioid peptides (EOPs), but acts via its own receptor, ORL1, which has yet to be characterized in ovine tissue. Neurons containing RFRP3 are found only in the region adjacent to the third ventricle within the PVN and DMH.[95,96] RFRP3-positive boutons contact GnRH neurons in the POA[75] and β-endorphin- and NPY-containing cell bodies in the ARC,[78] but the one study that examined expression of the putative RFRP3 receptor in sheep (by in situ hybridization) found little expression in the ARC, and none in the POA.[95] As noted earlier, no studies have looked for the presence of steroid receptors in ovine RFRP3 neurons at this time.

The location of steroid receptors and determination of relevant neural circuitry lay the anatomical foundation for analysis of the feedback actions of gonadal steroids, but provide no information on the function of these systems. The latter requires determination of the actions of the neurotransmitters using receptor agonists and antagonists, and of changes in neuronal function under different physiological conditions. Neuronal function is usually assessed in one of three ways: (1) measurement of mRNA (RT–PCR and in situ hybridization) and/or protein (immunocytochemistry) expression for peptide transmitters, or more indirect indices (e.g., transporters or synthetic enzymes) for the smaller neurotransmitters; (2) monitoring expression of the early immediate gene product, Fos, which has been used extensively as an index of increased activity and/or other intracellular signaling molecules; or (3) measurement of transmitter concentrations in samples collected by push–pull perfusion or microdialysis in the vicinity of nerve terminals.

Based on the effects of receptor agonists and/or antagonists, there is general agreement that dynorphin,[69] β-endorphin,[97] GABA,[98] somatostatin,[99] and OFQ inhibit,[58] while glutamate,[100] kisspeptin,[101] and NKB[102] stimulate, GnRH release in the ewe (Table 27.1). NPY usually inhibits GnRH,[103] but stimulatory effects have been reported under some circumstances.[104] Dopamine can have both inhibitory[105] and stimulatory[106] effects depending on the site of administration, and NE has either inhibitory or stimulatory effects depending on the endocrine condition of the ewe.[107,108] Because interest in RFRP3 is a fairly recent development, there have been only a few studies on its potential role, and the results to date are inconsistent. RFRP3 inhibited the response of ovine gonadotropes to GnRH in vitro,[96,109] and RFRP3 was found in the external zone of the median eminence[96] and in hypophysial portal blood.[110] Although in vivo inhibitory effects of intravenous (IV) administration of RFRP3 on LH secretion have been observed by one group,[96,110,111] the doses used produced RFRP3 concentrations far in excess of those found in portal blood.[110] Another group reported that RFRP3 given either IV (at similar doses to those used by others) or into the third ventricle had no inhibitory effects on LH secretion in ovariectomized (OVX) ewes.[112] Thus, any conclusions as to the physiological actions of RFRP3 in the ewe await further studies. The effects of galanin, agonists, or antagonists for its receptors have not been reported in ewes, and there is no direct evidence at this time that galanin or RFRP3 mediates any feedback effects of ovarian steroids in this species. Similarly, the possibility that glial cells play a role in steroid feedback, as they may in other species (see Chapter 11), has yet to be tested in sheep.

Control of Tonic GnRH and LH Secretion by Ovarian Steroids

Because tonic gonadotropin secretion always occurs in an episodic pattern, the negative-feedback actions of both estradiol and progesterone require direct or indirect interactions with the neural mechanisms driving pulsatile GnRH secretion. Therefore, we will first consider

recent information on these neural elements, and then discuss the effects of ovarian steroids on them.

THE GnRH PULSE GENERATOR[73,113]

The episodic pattern of GnRH release implies that the GnRH neurons controlling tonic LH secretion must function in synchrony, an inference supported by detailed analysis of pulsatile GnRH secretion rates.[114] Specifically, episodic GnRH secretion occurs essentially as a square wave, with very rapid onsets and offsets and no secretion between pulses (Figure 27.6), indicating tight coupling of the responsible GnRH neurons. Thus, a key first step in understanding the mechanisms of steroid negative feedback is to identify the neural systems responsible for the generation of GnRH pulses, and significant progress has been made during the last few years toward this goal. Early work that de-afferented input to the MBH with knife cuts indicated that the neural elements necessary for episodic LH secretion reside within this area in sheep,[115,116] as in other species (see Chapter 33), and bursts of multiunit electrical activity (MUA) that correlated with LH pulses were recorded in this area in OVX ewes.[117]

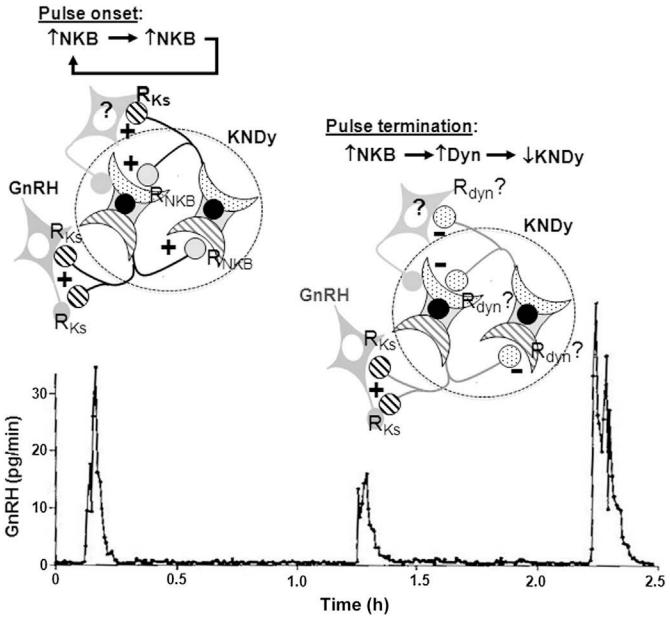

FIGURE 27.6 Model for role of KNDy neurons in driving episodic GnRH secretion. Representative GnRH pulse pattern is presented in the *bottom panel*, which depicts the GnRH secretion rate into hypophysial portal blood samples collected every 30 s for 2.5 h in OVX ewes. The model proposes that each pulse is triggered by an initial release of NKB (gray terminals) within the KNDy circuit, which increases the activity of these neurons. This initiates a positive-feedback loop that further increases NKB release onto KNDy neurons and kisspeptin (striped terminals) release that stimulates GnRH neurons, which have kisspeptin receptors (R_{Ks}) Within 1–2 min, the inhibitory effects of dynorphin (stippled terminals) within the KNDy circuit dominate, possibly due to a decrease in NK3R (R_{NKB}) or an increase in κ-receptor (R_{Dyn}) expression. Dynorphin inhibits KNDy neural activity, breaking the positive-feedback loop and suppressing kisspeptin and, thus, GnRH release to terminate the pulse; see the text for further details. Note that the pattern in each terminal indicates the biologically active transmitter (e.g., due to selective expression of postsynaptic receptors), not selective transport of that peptide to the terminal. *Source: Revised from Lehman et al.*[73]

More recently, several lines of evidence have led to the hypothesis that KNDy neurons in the ARC play an important role in driving episodic GnRH secretion,[73,113] including the following: (1) kisspeptin is needed for episodic LH secretion in OVX ewes,[105,118] (2) KNDy neurons form an extensive interconnected network capable of firing synchronously[119,120] and project to GnRH neurons in the ovine MBH and POA,[71] and (3) bursts in MUA activity that correlate with LH pulses in OVX goats appear to be recorded from KNDy neurons.[121] Moreover, a coherent model for the synchronous firing of KNDy neurons during episodic GnRH secretion has been developed that appears to be applicable to sheep and goats. This model postulates specific roles for each of the KNDy neuropeptides (Figure 27.6). First, the primary role of kisspeptin is to provide the output signal from KNDy neurons that drives each GnRH pulse; supporting evidence is that (1) GnRH neurons contain KISS1R (kisspeptin receptor, aka GPR54),[122] (2) KNDy neurons do not,[80] and (3) exogenous kisspeptin increased LH secretion without altering MUA in the ARC.[121] Second, the stimulatory effects of NKB on KNDy cells trigger a positive-feedback loop within the KNDy network to initiate each GnRH pulse; supporting evidence is that (1) KNDy neurons contain NK3R (tachykinin NK3 receptor), while GnRH neurons do not[123]; (2) intracerebroventricular (ICV) administration of an NK3R agonist increased MUA activity[124] and Fos expression in KNDy neurons[125]; and (3) local administration of an NK3R antagonist to the ARC disrupted episodic LH secretion.[126] Third, increased release of dynorphin dampens and eventually terminates KNDy neural activity and thus each GnRH pulse; supporting evidence is that (1) IV infusion of naloxone increased GnRH pulse amplitude and prolonged pulse duration,[127] (2) ICV administration of a κ-opioid receptor antagonist increased the frequency of MUA in the ARC,[124] and (3) the same κ-opioid receptor antagonist increased LH pulse frequency when given locally into the ARC.[126] The recent report that local administration of a KISS1R antagonist to the ARC of OVX ewes decreased LH pulse frequency indicates that endogenous kisspeptin also has effects on the activity of the KNDy neural network.[126] Because these actions must be via non-KNDy KISS1R-containing neurons found in the ARC,[80] we have modified our original proposal to include an unidentified interneuron that can interact with this circuit (Figure 27.6).

The possible interaction of specific GnRH neurons with this putative pulse generator are still under investigation, but GnRH neurons within the MBH are likely to be its

primary target, based on knife cut data[115] and the selective expression of Fos in MBH GnRH neurons when episodic LH secretion was stimulated.[46] The latter contrasts with the LH surge, during which GnRH neurons in all areas were activated (Figure 27.5).[47] It has been suggested, based on systemic administration of a GnRH receptor antagonist, that ultrashort loop feedback may contribute to the generation of GnRH pulses,[128] but other data in sheep are not consistent with this hypothesis,[129] and local administration of acyline to the ARC had no effect on pulsatile LH release in OVX ewes.[126] Thus, at this time it appears as if the activity of the three KNDy neuropeptides may be sufficient to largely account for GnRH pulse generation in sheep. Interestingly, two of these peptides have also been implicated in the feedback actions of ovarian steroids.

ACTIONS OF ESTRADIOL AND PROGESTERONE

Careful replacement studies reproducing normal progesterone and estradiol concentrations in acutely OVX ewes produced tonic LH concentrations in steroid-treated OVX animals that were virtually identical to those seen during the normal estrous cycle, while treatment with progesterone or estradiol alone modestly reduced tonic LH secretion, but not to normal levels.[130] Thus, estradiol and progesterone are both necessary and sufficient to account for the pattern and mean concentrations of tonic LH during the ovine estrous cycle. Quantitatively, progesterone is more important than estradiol because the latter is a weak negative-feedback hormone during the breeding season.[131] Progesterone produces a dose-dependent inhibition of LH and GnRH pulse frequency, while estradiol inhibits GnRH and LH pulse amplitude, but also stimulates pulse frequency.[107] Each of these individual actions is dose dependent, but because they tend to offset each other, basal estradiol concentrations are as effective as peak concentrations in suppressing mean LH. Estradiol also enhances the inhibitory actions of progesterone, but this action is not dose dependent.[107] Consequently, the inhibitory effects of estradiol are relatively constant throughout the cycle, and tonic LH secretion and GnRH–LH pulse frequency are inversely correlated with progesterone concentrations during the estrous cycle (Figures 27.1 and 27.2).

MECHANISMS MEDIATING PROGESTERONE NEGATIVE FEEDBACK[107]

Progesterone has one primary negative-feedback action: inhibition of GnRH pulse frequency. Although progesterone can have inhibitory effects directly on the pituitary,[1] the physiological relevance of these remains unclear, because pituitary effects were not always observed in vivo[132] and this steroid specifically inhibits LH pulse frequency, an action that reflects inhibition of GnRH pulse frequency. There is some evidence that progesterone increased GABA[133] and decreased glutamate[134] release in the POA, but knife cuts between the POA and MBH did not disrupt progesterone negative feedback,[107] so these systems probably do not play a critical role in the inhibitory actions of progesterone. Progesterone also partially inhibited *Kiss1* mRNA expression in the ARC based on in situ hybridization data,[135] but this has not been confirmed using qRT–PCR (Figure 27.7). Thus, the

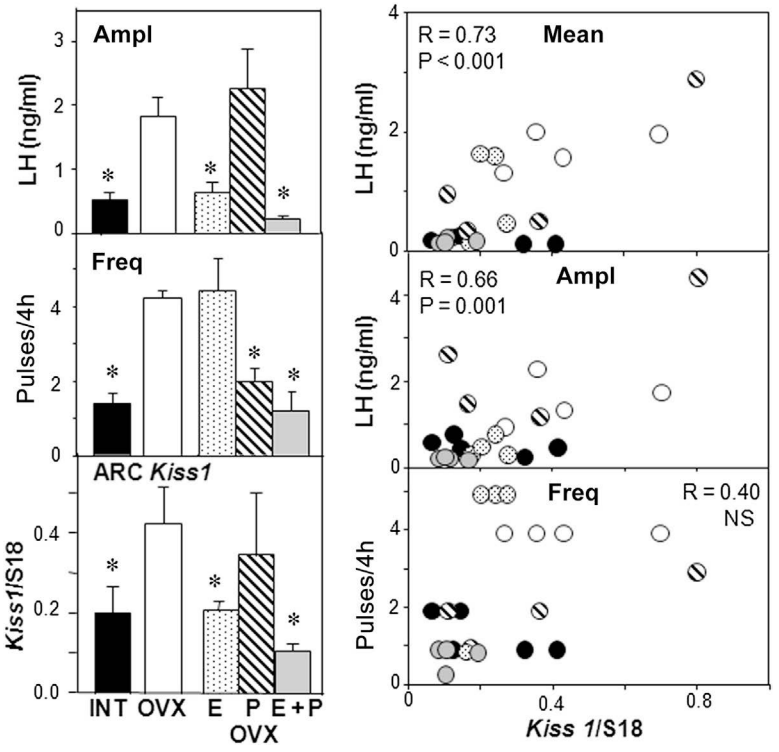

FIGURE 27.7 Effects of ovarian steroids on episodic LH secretion and *Kiss1* mRNA expression in the caudal ARC of ewes. Ewes ($n = 4$–5/group) were ovariectomized (OVX) and given either no implants (clear symbols) or implants that produced luteal-phase levels of estradiol (stippled symbols), progesterone (striped symbols), or both (shaded symbols). Eight days later, LH pulse patterns were monitored for 4 h, and tissue was immediately collected and frozen. Samples from ovary-intact (INT) luteal-phase ewes (black symbols) were collected at the same time. The caudal ARC was then microdissected, mRNA extracted, and *Kiss1* mRNA determined by qRT–PCR. Bars on the right depict mean ± standard error of the mean values for LH pulse amplitude (*top panel*), LH pulse frequency (*middle panel*), and *Kiss1* mRNA (*bottom panel*). *$P < 0.05$ versus OVX. Right panels present correlations of the mean LH (*top*), LH pulse amplitude (*middle*), and LH pulse frequency (*bottom*) with *Kiss1* mRNA expression in individual ewes. *Source: From Goodman RL, Rao A, Smith JT, Clarke IJ. Negative feedback control of Kiss-1 gene expression by estradiol and progesterone. Program: 1st World Congress on Kisspeptin Signaling in the Brain, Cordoba, Spain, 2008.*

general consensus is that EOPs are the primary mediators of progesterone negative feedback because several studies have demonstrated that EOP antagonists increased LH (and GnRH) pulse frequency in the presence, but not the absence, of progesterone.[1,107] More recent work points to dynorphin from KNDy neurons as the EOP mediating the negative-feedback actions of progesterone: (1) all KNDy neurons contain PR,[64] the receptor mediating progesterone negative feedback[136]; (2) local administration of a κ-receptor antagonist to the MBH increased LH pulse frequency in luteal phase ewes[69]; (3) OVX decreased expression of pre-prodynorphin mRNA in the ARC[137]; and (4) local microimplantation of RU486 into the region containing KNDy neurons blocked the negative-feedback action of systemic progesterone.[138] It should be noted, however, that microimplants containing progesterone had no effect when placed in the region containing KNDy neurons,[138] indicating that other neural systems act in concert with KNDy neurons to mediate progesterone negative feedback. Both dynorphin[69] and β-endorphin[97] can act in the POA to inhibit LH pulse frequency during the luteal phase but, as noted earlier, data from de-afferentation studies indicate that these other systems are likely in the MBH.[107] One possible system is the OFQ-containing neurons concentrated in the rostral ARC, which contain ERα and PR,[74] because an antagonist to the OFQ receptor increased LH secretion in OVX ewes treated with estradiol plus progesterone, but not those treated only with estradiol.[74]

MECHANISMS OF ESTRADIOL NEGATIVE FEEDBACK[107]

These are much more complex than those of progesterone because estradiol produces four different effects on episodic GnRH–LH secretion. During the luteal phase, it increases the ability of progesterone to inhibit GnRH pulse frequency.[136,139] During the follicular phase, estradiol (1) decreases GnRH and LH pulse amplitude,[140] (2) increases pulse frequency,[140,141] and (3) alters the shape of GnRH pulses and increases GnRH release between pulses.[142] The mechanisms underlying these actions are not well understood, in part because direct assessments of GnRH secretion are necessary to monitor most of them.

The primary negative-feedback action of estradiol is to inhibit LH pulse amplitude. Most evidence indicates that this reflects estradiol actions at both the pituitary and hypothalamus. Acutely (within a few hours), estradiol treatment decreased the response of the pituitary to GnRH, both in vivo and in vitro,[143] and completely suppressed LH pulses without disrupting GnRH secretion into the portal circulation under some circumstances.[1] The cellular mechanisms of this inhibitory action have not been completely elucidated, but early work demonstrated that estradiol acutely decreased expression of mRNA for the α and LH-β subunits.[143] More recent work has implicated membrane estrogen receptors in the acute negative-feedback actions of estradiol.[144–146] Binding of estradiol to these receptors activated the same second messenger systems as GnRH, but inhibited GnRH-induced increases in intracellular Ca^{++} concentrations,[147] so the latter most likely accounts for the acute negative-feedback actions of this steroid. Estradiol-induced increases in GnRH pulse frequency may also indirectly decrease LH pulse amplitude, because increasing the frequency of GnRH administration decreased LH pulse amplitude in response to each GnRH injection.[1,143,148]

Estradiol also decreased the amount of GnRH released per pulse,[142] an action that may occur in both the POA and MBH.[1] Of the 12 neural systems identified to contain ERα and project to GnRH neurons (Table 27.1), the possible roles of EOP, GABA, NE, and kisspeptin in estrogen negative feedback have been examined.[107] Although initial work monitoring LH pulse amplitude indicated that EOPs mediate this action of estradiol, direct measurement of GnRH ruled out a role for EOPs,[127] and it is unlikely that GABA mediates estrogen negative feedback.[98] There is evidence that NE may mediate this action of estradiol because estradiol rapidly induced Fos in A1 NE neurons[84] and microimplants of an α-adrenergic antagonist into the POA increased LH pulse amplitude in the presence, but not the absence, of estradiol.[107] On the other hand, knife cut data point to the MBH as the most important site of estrogen negative feedback,[115] and more recent studies have implicated KNDy neurons in this action. Specifically, OVX increased, and estradiol treatment decreased, kisspeptin peptide and mRNA expression in the ARC,[75,135] and OVX increased Fos expression in these neurons.[149] Moreover, a KISS1R antagonist decreased LH pulse amplitude when given ICV to OVX ewes[118] and kisspeptin mRNA expression in the caudal ARC, measured by qRT–PCR, correlated with LH pulse amplitude, but not pulse frequency, under a variety of endocrine conditions (Figure 27.7). Thus, KNDy neurons may mediate the negative-feedback actions of both estradiol and progesterone, but do so via different neurotransmitters. This hypothesis is consistent with the proposed roles of kisspeptin and dynorphin in the generation of GnRH pulses. If kisspeptin is primarily the output signal, then inhibition of kisspeptin expression would be expected to inhibit GnRH pulse amplitude. Likewise, if dynorphin terminates each GnRH pulse and prevents activity between pulses, then a progesterone-induced increase in dynorphin would inhibit pulse frequency.

Little is known about how estrogen exerts its other three actions on pulsatile GnRH secretion in breeding-season ewes. The ability of estradiol to increase progesterone inhibition of GnRH pulse frequency most likely involves dynorphin in KNDy neurons that contain both ERα[72] and PR,[64] and could reflect the well-established ability of this steroid to increase expression of PR. The

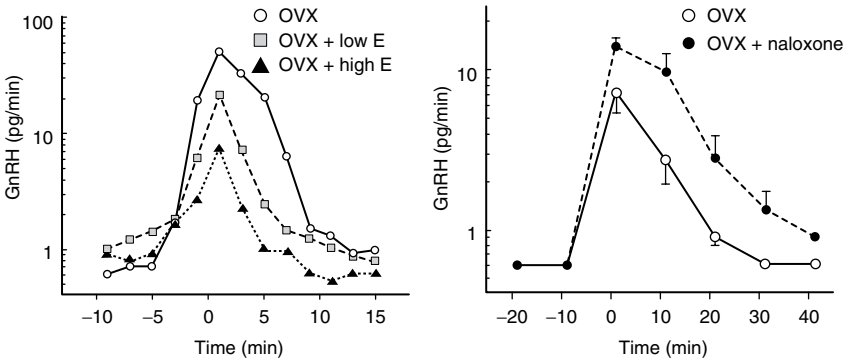

FIGURE 27.8 Effects of estradiol (E) and an EOP antagonist (naloxone) on the shape of GnRH pulses in OVX ewes. *Left panel*: Mean GnRH secretion rate, normalized to the peak of each pulse, in samples collected every 2 min from acutely OVX ewes treated with either no steroid or estradiol implants to produce luteal-phase (low E) or peak follicular-phase (high E) concentrations of E. *Right panel*: Mean GnRH secretion rate, normalized to the peak of each pulse, in samples collected every 10 min from ewes OVX 2 weeks earlier, before (OVX) and during (OVX + naloxone) intravenous infusion of naloxone. Note the difference in axes between two panels. *Source: Reprinted from Goodman et al.*[107]

mechanisms by which estradiol increases GnRH pulse frequency are completely unknown. It is unlikely that they involve EOPs or NE because antagonists to these neurotransmitters had no effect on pulse frequency in OVX+E ewes.[107,127] On the other hand, EOPs may be involved in the ability of estradiol to alter GnRH pulse shape because naloxone and estradiol produced opposite effects on GnRH pulse shape (Figure 27.8). This raises the possibility that the increase in β-endorphin release that occurs in the follicular phase[150,151] may mediate effects of estradiol on GnRH pulse shape. In contrast, both estradiol and naloxone increased the release of GnRH between pulses, so that this action of estradiol cannot be due to an increase in EOP. Finally, as discussed in the next section, these changes in GnRH pulse shape and in release between pulses are greatly magnified at the start of the preovulatory GnRH surge[18] so that these effects of estradiol most likely reflect positive, rather than negative, feedback actions of this steroid.

Control of the GnRH and LH Surges

HORMONAL CONTROL

It is now well established that the follicular-phase estradiol rise is the ovarian signal that induces the preovulatory surges of GnRH and LH,[1] while neither progesterone nor PR acutely contributes to the LH surge because (1) circulating progesterone concentrations do not increase during the periovulatory period of the ovine estrous cycle[14]; and (2) the PR antagonist, RU486, does not block the estradiol-induced surges of GnRH and LH in the ewe.[152] Induction of the LH surge does not require the full preovulatory estradiol rise[153]; an increase to midfollicular phase concentrations is sufficient. However, greater amounts of estradiol do increase the magnitude of the LH surge[153] and synchronize it with the onset of estrus,[1] so peak estradiol concentrations are required to mimic events during the estrous cycle. The most obvious action of progesterone is that chronically elevated concentrations block the ability of estradiol to induce an LH–GnRH surge.[130,152] Progesterone pretreatment and withdrawal also delay the onset of the GnRH–LH surge,[130,154] contributing to its synchronization with estrus, and augment the amplitude of the GnRH surge.[155]

MECHANISMS OF ACTION OF ESTRADIOL

The sites of estradiol action in triggering the LH surge include both the hypothalamus and pituitary. The amount of LH released in response to exogenous GnRH is maximal just before the onset of the LH surge,[143] and studies in which endogenous GnRH secretion was blocked and replaced with exogenous GnRH pulses have demonstrated that approximately 10–20% of the surge can be accounted for by estrogen actions at the pituitary.[1,143] The stimulatory effects of estradiol on the gonadotrope appear to be multifaceted and mediated primarily by nuclear ER. Acting in concert with increased GnRH pulse frequency, estradiol increased expression of both ERα[156] and GnRH receptors[132] in gonadotropes, both of which were maximal during the late-follicular phase.[51,132] Estradiol also increased the synthesis of LH and the number of Ca^{++} channels, and enhanced the movement of secretory granules toward the cell membrane.[1,143] All of these actions enhance pituitary responsiveness to GnRH, but the relative importance of each remains to be determined.

Direct measurements of GnRH have clearly demonstrated that estradiol produces a dramatic increase in mean GnRH release and alters the pattern of GnRH secretion so that it moves progressively from episodic to continuous.[17,18,142] As illustrated in Figure 27.9, GnRH secretion between pulses begins to increase just prior to the start of the LH surge; there is then a dramatic increase in both GnRH pulse amplitude and interpulse

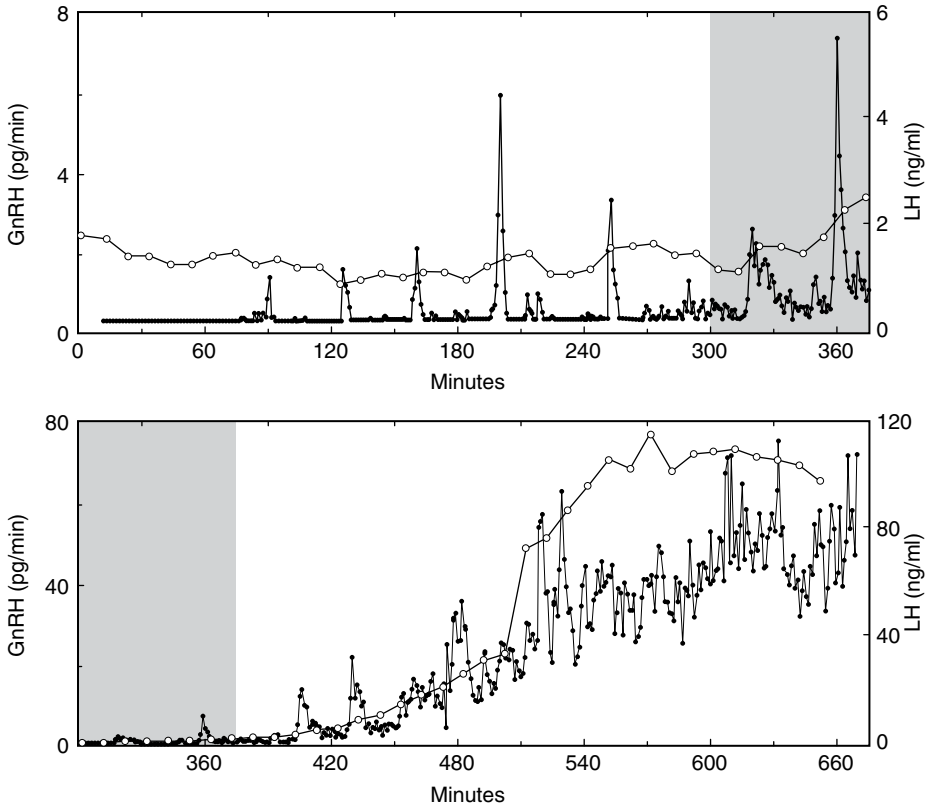

FIGURE 27.9 Time course of changes in the pattern of GnRH secretion leading up to and during the estradiol-induced preovulatory surges of GnRH and LH. GnRH secretion rates (solid circles) monitored every minute and peripheral LH concentrations (open circles) monitored every 10 min in an individual ewe are presented. Note the differences in the *y*-axis between the top and bottom panels. The shaded area depicts the time of overlap of values from the top and bottom graphs. *Source: Reproduced from Evans et al.[18] (Copyright 1995, The Endocrine Society).*

GnRH secretion during the ascending phase, which eventually leads to sustained erratic GnRH release during peak secretion. The mechanisms by which estradiol produces these effects are still poorly understood, but two questions have been resolved. First, studies using local administration of estradiol have demonstrated that it acts in the VMN–ARC region to induce the surge.[157] Second, there is no anatomically distinct subpopulation of GnRH neurons involved; approximately 40% of GnRH neurons in all hypothalamic areas (Figure 27.5) expressed Fos during the surge.[47] These data are consistent with the observation that knife cuts between the POA–AHA, where most of the GnRH perikarya are found, and the MBH decreased the amplitude, but did not completely block, the estrogen-induced LH surge.[115]

Early work implicated seven different neurotransmitters in the control of the GnRH surge, while more recent work has focused on the role of kisspeptin and NKB (Table 27.2). In analyzing this information, we have made use of a conceptual model[152] that divides the surge induction process into three stages: (a) activation, a period lasting between 4 and 14 h, during which estradiol must be present; (b) transmission, which lasts for 6–8 h and does not require the presence of estradiol; and (c) GnRH release, which also does not require the presence of estradiol (Figure 27.10). Of the nine potential transmitters, four have received significant attention: the inhibitory actions of β-endorphin and the stimulatory actions of NE, kisspeptin, and NKB. Levels of mRNA for the precursor of β-endorphin decreased during the activation stage,[160] while concentrations of β-endorphin in the median eminence increased during the transmission phase,[150,151] and then fell during the GnRH surge.[150] This pattern of release raises the possibilities that withdrawal of EOP inhibition contributes to the onset of the surge and EOP agonists did delay the surge.[162,163] However, EOP antagonists did not advance the surge,[162,172] so an EOP brake is unlikely to be the last limiting factor before the onset of GnRH release. Estrogen treatment acutely stimulated A1 NE neurons[84] and there is evidence for increased NE release in the POA during the transmission phase,[164,167] and in the median eminence during the GnRH surge.[150] However, an α-adrenergic antagonist did not consistently block the estrogen-induced LH surge,[158] so this transmitter may play only a facilitating role.

Kisspeptin contributes to, but is not solely responsible for, the GnRH surge in sheep because a KISS1R antagonist was able to reduce the LH surge by only approximately 50%,[80] even though it completely inhibited LH secretion in OVX ewes[80,105] and blocked the LH surge in rats.[173] However, which kisspeptin neurons (POA or KNDy) are responsible remains under debate. Estrogen stimulated kisspeptin expression in the POA,[75] and levels of *Kiss1* mRNA in these neurons increased during the follicular phase.[122] There is also agreement that Fos

TABLE 27.2 Evidence for Roles of Transmitters Implicated in Induction of GnRH Surge

Transmitter	Activation Phase (Estradiol Required)	Transmission Phase (Estradiol Not Required)	GnRH Release (Estradiol Not Required)	Caveates
Dopamine		Antagonist blocks[106,158]	Increased turnover in VMH[106]	Agonist did not advance[159]
β-endorphin (ARC)	Decreased mRNA[a,160] No change in Fos[161]	Increased in ME[b,150,151] Agonist delays[162,163]	Fall in ME[b,150]	Antagonist did not advance[162]
Glutamate (ARC)	Increased Fos[61]			Limited data
GABA	Increased release in POA[164]	Gradual decline in POA[164]		Limited data
Kisspeptin (POA)		Increased mRNA[a,122] Antagonist reduced amplitude[80]	Increased Fos[149,165]	Antagonist did not completely block[80]
Kisspeptin (ARC KNDy)	Increased Fos[122]	Increased mRNA[a] in some areas[122,149,166] Antagonist reduced amplitude[80]	Increased Fos[149]	Antagonist did not completely block[80]
Norepinephrine (A1 cells)	Increased Fos[84]	Increased pulses[164] and DBH[c] release in POA[167]	Increased in ME[b,150]	Antagonist did not consistently block[158]
Neurokinin B (ARC KNDy)	Decreased mRNA[a,168] Increased Fos[122]	No change in mRNA[a,65] Antagonist in RCh reduced amplitude[169]	Increased Fos[149] No change in mRNA[a,65]	Agonist induced surge[102] Antagonist did not completely block[169]
Neuropeptide Y (ARC)	No change in Fos[161] or mRNA[a,160]	Increased in ME[b,104]	Increased in ME[b,104]	Inconsistent effects of agonist[104,170] and antibodies[1]
Somatostatin (VMN)	Increased mRNA[a,99] Fos: No change at 1 h,[161] and increased at 6 h[171]		Increased Fos[66]	Limited data

[a] *mRNA for precursor of neuropeptide.*
[b] *ME: median eminence.*
[c] *DBH (dopamine-β-hydroxylase) is stored in NE-containing secretory vesicles.*

expression in this POA population increases at the time of the LH surge,[149,165] so this population likely participates in the secretory phase of estrogen action. There are two reports of increased Fos expression in KNDy neurons at the time of the surge,[149,174] and Kiss expression was also elevated in some of these neurons late in the follicular phase.[122,149,166] An increase in Fos was not observed in KNDy neurons in a third study,[165] possibly for technical reasons.[149] KNDy, but not POA kisspeptin, neurons were also activated 1 h after a surge-inducing estrogen injection,[122] so they are most likely involved in both the activation and release phases of the GnRH surge.

Initial interest in the possible role of NKB in the surge was dampened by observations that mRNA for the NKB precursor did not increase before or during the estrogen-induced surge,[65] and that estradiol acutely inhibited levels of this mRNA.[168] However, NKB in KNDy neurons may be involved because these neurons express Fos at the time of the LH surge[149] and local administration of the NK3R agonist, senktide, to the retrochiasmatic area (RCh) induced a surge-like LH pattern in ewes.[102,169] Moreover, recent evidence that local administration of a NK3R antagonist to the RCh decreased the amplitude of the estrogen-induced LH surge[169] provides strong evidence that NKB actions in this region are another component of the neural mechanisms responsible for the preovulatory GnRH surge. The NKB input to this region most likely arises from KNDy cells, and we have preliminary data that RCh neurons provide input to KNDy neurons, so this reciprocal circuit may be important during the transmission or secretory phases of estrogen positive feedback.

Of the other five neurotransmitters, two of them (glutamate[61] and GABA[164]) have been implicated in only one report (Table 27.2), and no consistent picture has emerged for NPY. Agonists to NPY receptors have been reported to advance[104] and delay the LH surge,[170] and NPY antisera can either delay or advance the surge.[1] Another two (DA and somatostatin) may be involved in estrogen actions in the VMN. Dopaminergic activity increased in the VMN coincident with the surge, and blockade of DA (and thus also NE) synthesis in this area delayed the surge.[106] These results are consistent with the early report that systemic administration of a DA D2 receptor

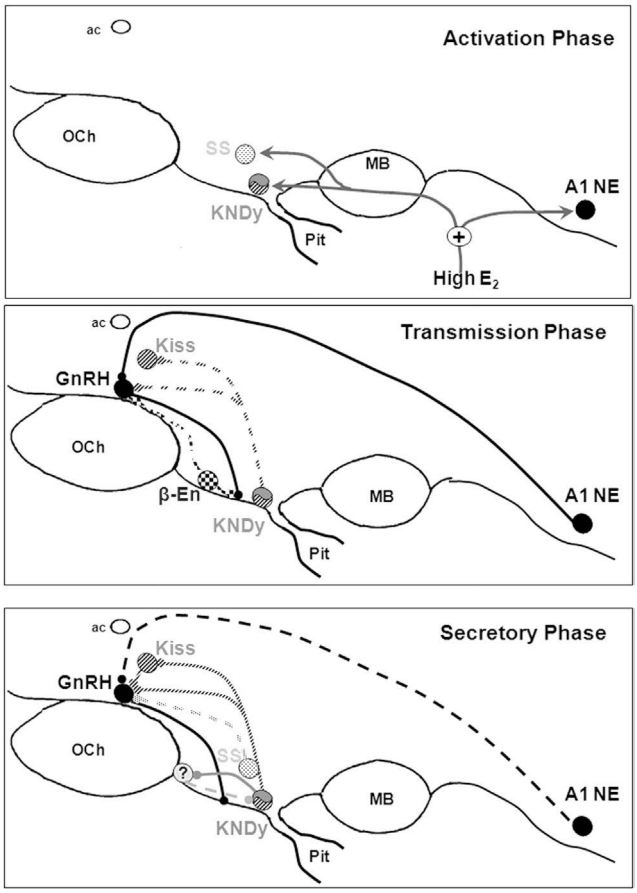

FIGURE 27.10 Neural systems postulated to participate in the estrogen-induced LH surge in the ewe. Note that solid connections are supported by direct evidence, and dashed ones by indirect evidence. This model proposes that these mechanisms are activated by estradiol acting on ARC KNDy and VMH somatostatin (SS), and, possibly, A1 noradrenergic (NE) neurons (*top panel*). During the transmission phase (*middle panel*), stimulatory NE and possibly kisspeptin inputs to GnRH neurons are counterbalanced by the inhibitory effects of β-endorphin at GnRH cell bodies and terminals. Sometime after β-endorphin release falls, GnRH secretion is initiated by kisspeptin from KNDy and POA kisspeptin neurons and maintained by a positive-feedback loop between KNDy and NK3R-positive neurons in the RCh (*bottom panel*). NE and somatostatin may also contribute to the stimulation of GnRH neurons at this time. Note that for simplicity, two aspects of this system have not been depicted here: (1) GnRH neurons in the AHA and MBH that participate in the surge, and (2) stimulatory inputs to GnRH terminals in the median eminence.

(D2R) antagonist disrupted the surge,[158] but not with the report that administration of a D2R agonist to the VMN during the transmission phase failed to advance the surge.[159] Estrogen treatment increased somatostatin mRNA levels at 4 h[99] and Fos expression at 6 h[171] (but not 1 h post injection[161]) in the VMN, and induced Fos in 81% of these neurons during the GnRH surge.[66]

In summary, one can propose the following speculative model for the estrogen-induced GnRH surge in the ewe (Figure 27.10). Estradiol acts on ARC kisspeptin neurons, and probably VMN somatostatin and A1 NE neurons, to activate the surge mechanism. During the transmission phase, there is an increase in stimulatory NE input to GnRH neurons, but this is balanced by an increase in inhibition by β-endorphin. At sometime during or after removal of the EOP brake, the reciprocal circuit between KNDy cells and NK3R-containing neurons in the RCh is activated and soon acts as a positive-feedback loop to maintain the kisspeptin release from KNDy neurons that drives GnRH secretion during the surge. These primary inputs are supplemented by kisspeptin from POA neurons, somatostatin from the VMN (driven by local DA release), and NE from the A1 in either a redundant or cooperative manner.

MECHANISM OF ACTION OF PROGESTERONE[152]

Much less is known about the mechanisms of the two actions of progesterone. The mechanism of the priming action of progesterone pretreatment that increases the amplitude of the GnRH surge has not been investigated extensively, probably because it was discovered fairly recently and is not reflected in the preovulatory LH surge, so GnRH must be measured.[155] Progesterone pretreatment did not increase Fos expression in GnRH neurons during the estradiol-induced GnRH surge, but did increase Fos in non-GnRH POA neurons.[175] This raises the possibility that the latter neurons may provide stimulatory input that augments GnRH release, although they also could be involved in behavioral estrus (discussed further in this chapter).

The ability of progesterone to block the LH surge is due to inhibition of the GnRH surge, not inhibitory effects of this steroid at the pituitary.[176] It is mediated by the classical PR[152,177] and prevented both the activation[178] and transmission[179] phases of the estradiol-induced surge. Progesterone treatment throughout these two phases blocked Fos expression in GnRH neurons, but did not consistently affect Fos expression in other POA neurons.[175] This is consistent with the report that progesterone acted in the VMN, not the POA, to block the LH surge.[180] Progesterone did inhibit Fos in the VMN during activation and in the ARC at the end of the transmission phase.[181] Which neurotransmitters are involved in this action of progesterone remains unclear. Studies with the EOP antagonist, naloxone,[182,183] indicated that EOPs are not involved in progesterone blockade of activation and play only a minor role in the disruption of transmission.

Control of FSH Secretion

The only new information on the control of FSH secretion since the previous version of this chapter (in the third edition of this book) comes from a few studies on the role of anterior pituitary paracrine factors, so we will briefly summarize the discussion that was presented in that previous version,[1] and then consider the latter in more detail.

The preovulatory FSH surge is controlled by the same neural mechanisms that produce the coincident LH surge: the follicular-phase rise in estradiol secretion acting in the brain triggers the FSH surge, which can be blocked by a neural action of progesterone. Tonic FSH secretion throughout the remainder of the estrous cycle is controlled largely by estradiol and inhibin secretion from the ovary and paracrine effects of activin within the pituitary. Based on the patterns of estradiol and inhibin during the cycle (Figure 27.1), one can infer that the follicular-phase estradiol rise is responsible for the fall in FSH concentrations at this time, while changes in inhibin concentrations probably account for the "second FSH surge" on day 1 on the cycle.[11] It is less clear how ovarian hormones control the FSH fluctuations associated with follicular waves during the remainder of the cycle; as illustrated in Figure 27.3, estradiol and inhibin can account for changes in FSH associated with the first wave, but concentrations of these ovarian hormones did not change during the second wave in this study (probably because of decreasing LH release, as discussed later), so they cannot account for FSH changes seen during the second wave.[20] Similarly, periodic increases in FSH release observed in anestrus do not correlate with changes in either inhibin or estradiol.[30] There is one report of waves in FSH secretion occurring every 3–4 days in the absence of ovarian hormones,[184] but these observations need to be confirmed, and how such a rhythm could be generated is completely unknown. Evidence for a distinct hypothalamic FSH-releasing factor was reviewed in the third edition's version of this chapter, but no new information has been developed since then and interest in this possibility appears to have waned.

It is generally agreed that the ovine pituitary produces activin, inhibin, and follistatin, but the physiological functions of these locally produced peptides are less clear. Although several studies have demonstrated that inhibin can decrease FSH release in vitro,[1] no changes have been observed in hypophysial mRNA for inhibin receptors[185] or in local levels of mRNA or protein for the α-subunit of inhibin[186] in the pituitary at times during the estrous cycle when FSH secretion varies markedly. Similarly, pituitary inhibin-A expression did not change in response to variations in GnRH that stimulated FSH secretion,[187] and follistatin concentrations actually increased in response to a GnRH treatment that increased FSH release.[187] Activin can stimulate FSH synthesis and release in vitro,[188,189] and in vivo data support a physiological role for this peptide. The most consistent in vivo evidence comes from the strong positive correlations of FSHβ mRNA with the mRNA levels of three activin receptors[185] and of activin-βB (but not βA)[186] at the time of the second FSH surge. The mechanisms responsible for these changes in activin expression in the ovine pituitary remain to be determined, particularly in light of recent data that expression of activin B protein was unaffected by changes in GnRH pulse frequency.[187] There has been some recent work on the possible roles of bone morphogenetic proteins (BMPs) that are members of the same large protein family as activin and are found in the pituitary, but support for an important role for these peptides is not compelling. Early work indicated that BMP6 and BMP7 could stimulate FSH release,[190] but subsequent studies reported an inhibitory action of BMP4,[188] or that BMP2 and BMP6, but not BMP4, inhibited FSH release in vitro.[189] However, the inhibitory effects of BMP2 and BMP6 were observed at only one of the doses tested in the latter study. Moreover, a careful examination of expression of receptors and signaling molecules for BMPs found little change during the estrous cycle, and the authors concluded that it was unlikely that these proteins played an important role in regulation of FSH secretion in the ewe.[186] Thus, it appears at this time that activin is the only pituitary paracrine factor important to changes in FSH synthesis and secretion during the ovine estrous cycle.

Control of Estrous Behavior[5]

Hormonal Control

Under physiological conditions, both progesterone and estradiol are necessary for the induction of estrous behavior in the ewe.[5] Estradiol treatment alone can produce estrus, but in the absence of progesterone pretreatment, pharmacological doses are required.[1,5,130] Progesterone pretreatment for several days increases the sensitivity to estrogen and the intensity of both proceptive and receptive behavior.[191] There are breed differences in sensitivity to estradiol[192] but, in general, low concentrations can induce estrus in virtually all ewes, with higher concentrations decreasing the latency to estrus and increasing the proceptive activity of the ewe.[1,5,153,193]

The temporal relationship between progesterone and estradiol during the estrous cycle is also important for initiation of estrus. In contrast to the facilitating effects of pretreatment with progesterone, when progesterone was given simultaneously with estrogen it blocked estrous behavior,[5] and estrogen treatment was most effective if given a few days after progesterone withdrawal. Thus, the ideal steroid pattern for induction of estrus in the ewe is a prolonged period of elevated progesterone followed by a fall in progesterone and a rise in estradiol over the next few days, a pattern very similar to that observed during the ovine follicular phase (Figure 27.1). This pattern also synchronized estrus with the preovulatory LH surge; progesterone decreased the latency to estrus,[5] but increased that to the LH surge,[130,154] moving the two events closer together, and the full preovulatory E_2 rise completes this synchronization.[1] The fall in estradiol at the time of the LH surge is important for termination of

estrus, because prolonged estrogen treatment doubled its duration.[193] The physiological importance of both steroids is further emphasized by the absence of estrus at the transitions between the anestrous and breeding seasons.[1] At the start of the breeding season, the first ovulation is not accompanied by estrous behavior, because of the lack of progesterone priming. At the end of the breeding season, estrus does not occur after luteolysis, because estradiol concentrations fail to rise.

Neural Control

In contrast to the wealth of information on the hormonal control of estrous behavior in the ewe, the neural substrates for this behavior remain largely unknown. Based on lesions and local administration using microimplants,[194] estradiol acts in the VMN to induce estrous behavior.[1,5] Interestingly, progesterone increased expression of ERα in this region[195] which might explain the mechanism of progesterone priming. In contrast, progesterone microimplants blocked the ability of systemic estrogen treatment to induce sexual receptivity when placed in either the POA or VMN.[180] To date, three neurotransmitters have been implicated in the control of estrous behavior in the ewe: DA, GnRH, and oxytocin.[5] DA release in the VMN was stimulated by progesterone withdrawal, and then fell rapidly only if estradiol was administered to induce estrus.[196] These data led to the hypothesis that DA first acts in the VMN to stimulate the neural systems responsible for estrous behavior, but then inhibits this activity. This proposal is supported by the effects of local administration of a DA D2R agonist to the VMN, which increased the intensity of estrous behavior when given before systemic estradiol treatment, but decreased it when given after estradiol.[159] Interestingly, these treatments had no effect on the onset or duration of estrous behavior, indicating that other neurotransmitters are involved in triggering and terminating this behavior; both GnRH and oxytocin have been implicated in the latter. During the preovulatory GnRH surge, GnRH is released into both the portal circulation and the cerebrospinal fluid (CSF) in the third ventricle.[197] Administration of a GnRH antagonist into CSF during the latter part of the GnRH surge prematurely terminated estrous behavior, providing strong evidence that GnRH in CSF helps ensure that estrous behavior lasts for 36 h in the ewe.[198] Conversely, coitus-stimulated oxytocin release in the region of the VMN and infusion of oxytocin in this region suppressed receptivity.[5] This inhibitory effect of coitus-induced oxytocin release may account for the shortening of receptivity that occurs when ewes are exposed continuously to rams.[5]

Control of Ovarian Function

A complete review of the control of ovarian function in the ewe is beyond the scope of this chapter. Instead, this section will focus on factors responsible for follicular development, luteal development and regression, and hormone secretion throughout the ovine estrous cycle.

Control of Follicular Development[22]

Major workers in this field summarized the literature and proposed a model for follicular development in 1993, and have updated that model in a recent review.[22] In this section, we have used this review as a framework for our discussion, adding relevant newer data from ewes where appropriate. Movement from the primordial to primary and small preantral follicles (up to approximately 2 mm in diameter) occurs continuously throughout the estrous cycle because it is independent of gonadotropins. Instead, it is controlled by intrafollicular factors, primarily those coming from the oocyte. Atresia is low during these stages, but rates of atresia increase progressively as the follicles become more and more reliant on gonadotropic support. Under conditions of low concentrations of FSH and LH, thecal cells differentiate and express LH receptors, and follicles become responsive to gonadotropins. With continued growth and increases in LH and FSH, the gonadotropin-responsive follicles acquire aromatase and become gonadotropin dependent. Because of the requirement for gonadotropic support, gonadotropin-dependent follicles are susceptible to a high rate of atresia if that support is withdrawn, a characteristic that likely leads to follicular waves. If gonadotropin-dependent follicles continue to develop, they acquire LH receptors on granulosal cells and high levels of aromatase, and thus become potential ovulatory follicles. The latter will usually become atretic if an LH surge does not occur within about 72 h.

During the ovine estrous cycle, movement of follicles through their developmental trajectory is controlled by changes in concentrations of FSH and LH. During most of the luteal phase when LH pulse frequency is low, FSH plays a critical role because suppression of endogenous FSH peaks blocked emergence of follicular waves,[199] and exogenously produced FSH increments consistently initiated new follicular waves.[33,184] FSH concentrations simply have to be above a critical threshold for the development of gonadotropin-dependent follicles because neither fluctuations in the amplitude of FSH peaks during the luteal phase nor increases in the amplitude of an FSH peak affected the characteristics of the subsequent follicular wave.[21,184] Thus, a small rise in FSH above this threshold allows emergence of a cohort of follicles at the start of a follicular wave. As one or more of these become "potentially ovulatory follicles", they produce increasing amounts of estradiol and inhibin, and suppress FSH concentrations to below the threshold required by the other gonadotropin-dependent follicles so that they eventually become atretic. Thus, ablation of the largest follicle significantly lengthened the lifespan

of the second largest follicle in a monovulatory breed.[200] The secretory activity of the largest follicle may also control timing of the next follicular wave. According to this hypothesis, in the luteal phase these potentially ovulatory follicles eventually undergo atresia in the absence of an LH surge, which allows FSH concentrations to rise and initiate the next follicular wave. Although this is an attractive hypothesis, recent data do not appear to support it because an extra follicular wave, induced with exogenous FSH, did not delay the normal timing of the next endogenous increment in FSH secretion and follicular wave.[33] It should be noted, however, that inhibin, which appears to be important to the suppression of FSH,[200] was not measured in that study, and follicles in the extra wave produced abnormally low peak estradiol concentrations. The inability of ablation of all large follicles midway through a wave to advance the onset of the next wave also argues against this hypothesis, but estradiol concentrations were already declining at the time of ablation in that study.[200] Thus, there is now strong evidence for follicular dominance within a wave, but not that atresia of one wave is critical for the FSH increment that induces the next wave. Although the hypothesis that an ovarian-independent rhythm in FSH release drives follicular waves during the ovine luteal phase is intriguing,[33,184] more work is needed to rule out a role for inhibin and estradiol and to identify a mechanism for such a rhythm.

During the follicular phase of the cycle, the final stages of follicular development require both gonadotropins. During the early follicular phase, when LH pulse frequency is still relatively slow, elevated concentrations of FSH are required for the development and maintenance of potential ovulatory follicles. If these FSH concentrations were maintained throughout the follicular phase, they would be sufficient for the development of preovulatory follicles (Figure 27.11).[201] However, FSH concentrations fall as estradiol rises, so the preovulatory follicles must switch their dependence from FSH to LH during the follicular phase. Both the FSH induction of LH receptors on the granulosal cells of preovulatory follicles and the increasing LH pulse frequency seen in the follicular phase are critical for this switch, and thus ultimately for ovulation of a mature ovum. The importance of the latter is evident by the ability of high-frequency LH pulses produced exogenously in the luteal phase to maintain potentially ovulatory follicles[202] and the inverse correlation of progesterone concentrations and ovulation rates in Barbados blackbelly ewes.[203] It should be noted that episodic LH secretion is not essential; constant infusion of LH was capable of supporting ovulatory follicles in GnRH antagonist–treated[204] or prepubertal[205] ewes.

As noted elsewhere, only modest progress on our understanding of gonadotropic support of follicular development has occurred over the last decade.[22] In contrast, major new information is available on the molecular changes, oocyte–follicle interactions, and other intrafollicular factors involved in follicular development, including growth differentiation factor 9 (GDF9) and BMP15 from the oocyte, components of the insulin-like growth factor (IGF) system, and endothelial cell mitogens such as vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2). The reader is referred to Chapter 21, and two recent reviews for discussions of possible roles of intraovarian factors in follicular development.[22,206]

Control of Ovulation Rate[22]

Ovulation rate is influenced by hormonal, genetic, and environmental factors. One important hormonal influence is progesterone, with low concentrations in peripheral blood increasing ovulation rate, independent of nutritional management. In studies of the effects of low progesterone on the persistence of follicles, there was a trend toward an increased ovulation rate[27] and a prolific breed of ewes had lower concentrations of progesterone than a nonprolific breed.[26] Similarly, in a prolific breed of sheep, ovulation rate was negatively linearly correlated with concentrations of progesterone but lower progesterone did not increase lambs born.[203] Anestrous ewes pretreated to produce lower progesterone before ram introduction than seen in the luteal phase had a greater ovulation rate than ewes with ram introduction alone.[207] Consequently, treatment at progesterone withdrawal and ram introduction with low dosages of FSH, which had been shown to increase ovulation rate in anestrous ewes induced to ovulate with ram introduction, did not increase ovulation rate or litter size beyond that obtained in progesterone-pretreated ewes.[207]

There are several instances in which single-gene mutations in BMP15 in the oocyte; its receptor in the oocyte, granulosa, or corpora lutea; or GDF9 in the oocyte have a major influence on ovulation rate (reviewed by Montgomery et al.,[208] Fabre et al.,[209] and Otsuka et al.[210]). Immunization against either BMP15 or GDF9 increased ovulation rate in Romney ewes, with fewer single ovulations and more cases of three or greater ovulations,[211] but immunization against BMP15 did not increase response to a superovulatory treatment.[212] Six different sites of mutation have been identified in the BMP15 gene on the X chromosome, and they occur in five different breeds: two in Romney, two in Belclare, and one each in Cambridge, Lacaune, and Rasa Aragonesa. The Galway mutation in BMP15 occurs in Belclare and Cambridge, which also share a mutation in GDF9 on chromosome 5. The Icelandic Thoka has a different mutation in GDF9. The Booroola mutation in BMP receptor 1B, originally found in Merino on chromosome 6, has been identified in four other breeds. Finally, there is evidence for a maternally imprinted gene on the X chromosome, the

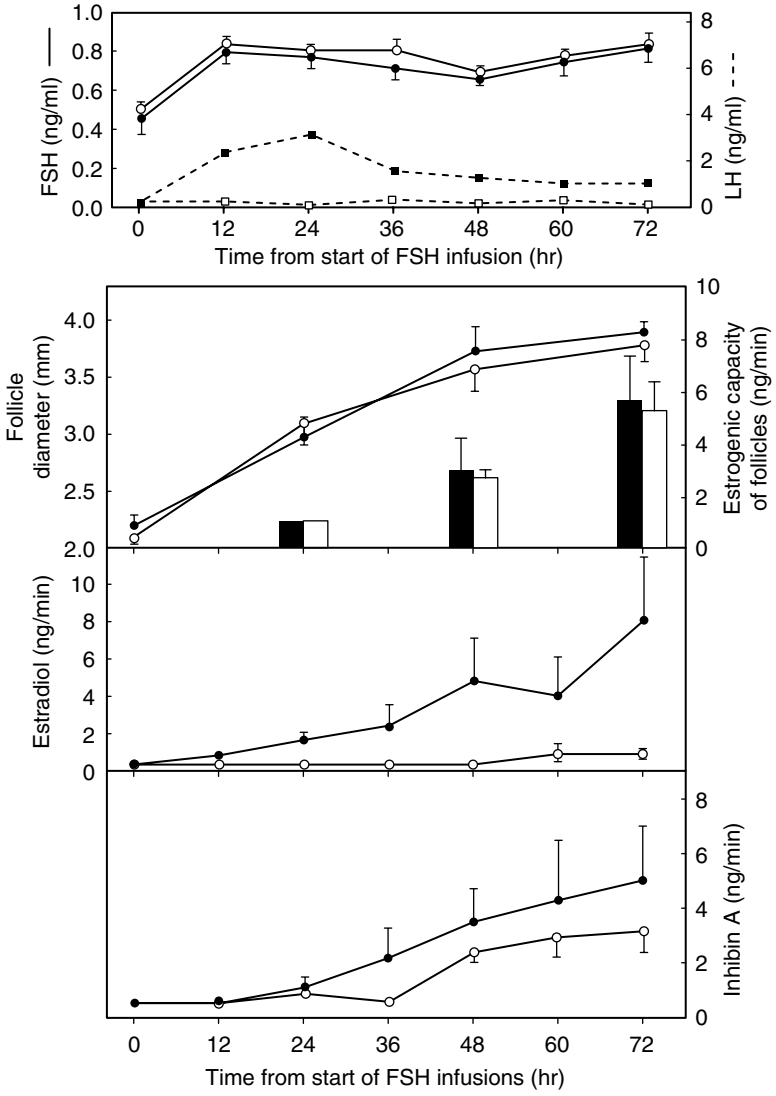

FIGURE 27.11 Effects of FSH, with and without LH, on the development of, and hormone secretion from, follicles in ewes with an ovary autotransplanted in the neck. Gonadotropin secretion was suppressed by long-term treatment with a GnRH antagonist. FSH was infused intravenously (IV) for 72 h, with (closed symbols) and without (open symbols) episodic LH injections (every 4 h). *Top panel* depicts FSH (circles) and LH (squares) concentrations; *bottom panels* present follicular diameter and secretion rates of estradiol and inhibin. Estrogenic capacity of the follicles (peak estradiol secretion rate in response to a single IV injection of LH) was determined daily. *Source: Data redrawn from Campbell et al.*[201]*; reproduced by permission of the Society for Endocrinology.*

Woodlands gene, which increased ovulation rate by 0.4 oocytes in the daughters of carrier rams.[213]

The genes related to BMP15 and GDF9 vary in the amount by which ovulation rate is increased by the effective allele (from one to 10 oocytes), and in physiological characteristics influenced by the gene. The heterozygous (single-copy) mutations in BMP15 and GDF9 described above increase ovulation rate, but homozygosity leads to sterility with no ovulation.[214,215] However, in Brazilian Santa Ines sheep with another polymorphism in GDF9, homozygosity increased ovulation rate by 82% and prolificacy by 58%.[216] Similarly, the Booroola gene ($FecB^B$) is maximally effective in increasing ovulation rate when homozygous. Crawford et al. found lower expression of mRNA for BMP15, but not of GDF9, in oocytes of homozygous Booroola ewes, and an earlier onset of responsiveness of the follicles to LH, which they proposed was similar to the effect of heterozygous BMP15 mutations.[217] The Booroola gene increases secretion of FSH, but most often the effects are in follicles; they have fewer granulosal cells, acquire LH receptors earlier, mature at a smaller size, and produce smaller corpora lutea than in noncarriers. Older ewes with $FecB^B$ maintained higher ovulation rates than noncarriers despite the fact that the numbers of developing follicles did not differ between genotypes, and concentrations of FSH increased and those of inhibin A decreased in older ewes, regardless of genotype.[208] Thus, the suggested action of the $FecB^B$ allele is to reduce the rate of follicular atresia. Current knowledge of the mechanisms of action of these genes and their mutations in sheep and other species has been reviewed.[22,210]

The effects of nutrition on ovulation rate have been recognized for almost a century and have been used extensively in attempts to increase fertility. Early work on nutritional "flushing" of ewes to increase the ovulation rate indicated that it required an increase in nutrition for at least the length of an estrous cycle to be

effective and that energy from a high-grain diet had a greater effect than protein from soybeans[218] when either or both were increased above a maintenance regimen. Coop[219] and others advanced the concept of different effects of static (due to body condition) and dynamic (temporary increases, i.e., "flushing") nutritional signals, a hypothesis that continues to provide a useful structure for interpreting contemporary data. A recent thorough review on nutritional influences on folliculogenesis and ovulation concluded that flushing effects occur mainly at the ovary.[22] Negative energy balance affects multiple levels within the hypothalamic–pituitary–ovarian axis,[22] but extremely severe restrictions are required to produce these effects.[1] The effects of flushing reflect regulatory mechanisms, not simply an increase in available energy, and recent work has focused on identification of systemic nutrient and metabolic signals and intrafollicular sensors and signaling pathways.[22] These have established that glucose and insulin act as mediators of short-term influences, while body condition is signaled primarily via the growth hormone–IGF system and leptin. These signals then have important regulatory roles in the oocyte and follicle. Notably, the oocyte influences its own fate (ovulation or loss via follicular atresia) through its effects on the follicle via BMP15 and GDF9, as described in this chapter. A useful model integrating nutritional and metabolic inputs that affect ovulation rate is provided in the review by Scaramuzzi et al.[22]

While the early work had indicated that flushing required nearly a cycle to have an effect, a more rapid action has recently been observed by synchronizing a follicular wave.[220] The first wave of an ovulatory cycle was synchronized among ewes in either high or low body condition with two injections of $PGF_{2\alpha}$ (7 days apart), and supplementation with lupin grain started (on day 0) during the growing phase of the postovulatory wave (2 days after the second $PGF_{2\alpha}$ injection); follicles from this wave were then allowed to ovulate by inducing luteolysis on day 5. Lupin supplementation for just 6 days in this model increased emergence of a second follicular wave 4 days later, and tended to increase the rate of ovulation of follicles in this wave in ewes in high (1.7 versus 1.3 ova) but not low (1.1 versus 1.2 ova) body condition. In three of seven supplemented high-condition ewes, ovulations occurred from both the first and second waves, despite the short interval for development of the second wave. Thus, nutritional effects may be possible in a shorter time frame than was previously thought.

Control of Follicular Hormone Secretion

Of the two major follicular hormones, the control of estradiol secretion has received considerably more attention than control of inhibin secretion, and several lines of evidence support the conclusion that LH pulses are the primary stimulator of estradiol release during the cycle. Early work demonstrated that (1) there is a positive correlation between tonic LH secretion and estradiol during the cycle, (2) an LH pulse precedes each episode of estradiol release into the ovarian vein, (3) blockade of LH secretion prevents the follicular-phase rise in estradiol, and (4) mimicking the follicular-phase LH increment in anestrous ewes produces a preovulatory-like estradiol rise in the presence of unchanging or decreasing FSH concentrations.[1] The hypothesis that LH was the sole driver of the preovulatory estradiol rise was challenged by a series of studies in the early 1990s using a GnRH agonist to suppress gonadotropin secretion.[221] In this model, FSH administration in vivo, even in the absence of LH pulses, stimulated follicular development and increased estradiol production by these follicles in vitro. However, in subsequent work using chronic treatment with a GnRH antagonist that markedly suppressed both gonadotropins, FSH infusion alone stimulated the growth of ovulatory follicles, but little or no estradiol secretion occurred (Figure 27.11).[201] When the follicular-phase pattern of episodic LH secretion was added to FSH, it had no effect on follicular growth but markedly stimulated steroid secretion. Thus, although FSH secretion clearly plays an important role in controlling follicular development and ovulation rate, it appears to be largely permissive for the preovulatory estradiol rise because FSH concentrations are declining at this time. Similarly, LH is most likely the primary hormonal driver for estradiol secretion during the luteal phase. This critical role for LH is illustrated by the different estradiol secretion patterns during the first and second follicular waves[20]; during the first wave, when LH pulse frequency is high (because of low progesterone), there is a progressive increase in estradiol secretion, but in the second wave when LH pulse frequency is suppressed there is no increase in estradiol secretion, despite the same rate of follicular growth (Figure 27.3). In addition to FSH and LH, progesterone can modulate estradiol secretion, and it does so by two mechanisms: (1) by inhibiting tonic LH secretion, progesterone indirectly suppresses estradiol production; and (2) progesterone directly inhibits the response of the follicle to exogenous LH.[1] The latter effect may account for the observation that higher LH pulses during the luteal phase produce similar or smaller estradiol increments than those during the follicular phase.

With the hormonal regulation of preovulatory estradiol rise largely resolved, more recent work has turned toward determining which intrafollicular factors control synthesis of this steroid. At this time, there is growing evidence for local stimulatory actions of IGF1,[22] inhibin A,[222] BMP2,[223] and BMP6[224] on estradiol synthesis, while leptin,[225] activin A and B,[42] and anti-Müllerian hormone[226] inhibit estradiol production. However, these data must be interpreted cautiously because some of these effects

may reflect an action on follicular development, and the relative importance of each factor remains unclear.

The hormonal control of inhibin A secretion has been more difficult to unravel, in part because secretion of this hormone is closely coupled to changes in follicular development, so independent hormonal effects on its release are difficult to identify. Early work, summarized in this chapter in the third edition of this volume, led to the conclusion that neither LH nor FSH stimulated inhibin secretion. Although exogenous FSH did increase inhibin production, this was thought to reflect its stimulation of follicular development, and additional LH treatment did not further augment inhibin release (Figure 27.11). However, more recent studies have provided strong evidence that FSH is a potent stimulator of inhibin secretion from follicles in vitro[222] and that an LH pulse frequency seen late in the follicular phase (once per hour) stimulates inhibin secretion in vivo.[204] In light of these stimulatory effects of LH and FSH, it seems likely that inhibin concentrations remain relatively stable during the follicular phase because FSH concentrations fall as LH concentrations rise. As noted earlier for estradiol, the absence of an increase in inhibin during the second follicular wave of the luteal phase (Figure 27.3) also argues for an important role for LH in stimulating inhibin secretion at this time of the cycle.

In contrast to the stimulatory effects of tonic LH secretion on estradiol production, the LH surge terminates estradiol secretion.[1] The LH surge appears to directly inhibit aromatase activity so that estradiol concentrations fall before any changes in androgen production are evident. Likewise, an ovulation-inducing dose of human chorionic gonadotropin inhibits inhibin secretion from the preovulatory follicle,[204] producing a fall in inhibin concentrations similar to that seen after the normal LH surge (Figure 27.1). Whether these changes reflect direct effects of the LH surge or indirect effects via luteinization remains to be determined.

Control of Luteal Progesterone Secretion[227,228]

As reviewed in detail in the previous versions of this chapter, the functional activity and demise of the corpus luteum during the ovine estrous cycle are controlled largely by luteotropic factors from the pituitary (LH and GH) and luteolytic factors from the uterus ($PGF_2\alpha$) and ovary (estradiol and $PGF_2\alpha$). Both LH and uterine $PGF_2\alpha$, in turn, are regulated by progesterone. Actions of these hormones on corpora lutea in sheep,[229] in cattle,[230] and on a comparative basis[231] have been reviewed recently and are addressed in Chapter 23. Here, we will focus on other new information raised in the last decade, with an emphasis on intraluteal factors.

LUTEAL DEVELOPMENT

The steroidogenic cells of the corpus luteum form by transformation of granulosal cells into the large cells and of thecal cells into the small cells; important studies of their characteristics were cited in the reviews by Niswender et al.[228,229] A key factor in luteal development is angiogenesis, because of the extensive vascular supply required for this active gland.[230] Cytokines involved include VEGF and FGF2. VEGF is a potent mitogen for vascular endothelial cells that stimulates migration of endothelial cells. VEGF mRNA was greatest in ovine corpora lutea early in the estrous cycle and least during luteal regression. VEGF protein was localized to capillary pericytes and vascular smooth muscle cells in the early corpus luteum. Because FGF2, which was localized to endothelial cells in the early corpus luteum, enhanced expression of endothelial platelet-derived growth factor receptor in pericytes and smooth muscle cells surrounding vasculature, Miyamoto et al.[230] concluded that FGF2 may be more important than VEGF in luteal angiogenesis in the cow. As pointed out in the previous edition's version of this chapter, estrogen, acting locally via ERα, may also play a role in activating secretion of progesterone by the early corpus luteum, which is consistent with the finding that blockade of aromatase delayed the initial rise in secretion of progesterone early in the estrous cycle.[232]

LUTEAL SUPPORT

Although LH is the primary luteotropic hormone during the ovine estrous cycle, it appears to play largely a permissive or supportive role in progesterone secretion. This conclusion comes in part from the inverse correlation of LH and progesterone concentrations during the cycle; because progesterone secretion is maximal when LH concentrations and LH pulse frequency are minimal, this gonadotropin is unlikely to be the primary factor controlling progesterone secretion rates (Figure 27.1). The relative independence of progestrone secretion from LH occurs because most progesterone secretion derives from large luteal cells that are unresponsive to LH, as they normally function at maximal capacity. Growth hormone (GH) interacts with LH to serve as a supplementary luteotropin in ruminants. The ovine corpus luteum expresses GH receptors and IGF-I and its receptors,[233,234] and treatment with a combination of LH and GH restored luteal weight to near normal in hypophysectomized ewes.[233] Treatment with LH restored concentrations of mRNA for GH receptor in the corpora lutea of the hypophysectomized ewes, while treatment with GH restored mRNA for IGF-I. However, neither mRNA for IGF-I nor for GH receptor changed after a luteolytic dose of $PGF_2\alpha$. Perhaps regulation of the action of GH through IGF-I is achieved by changes in the IGF binding protein 1, which was increased in response to $PGF_2\alpha$ in cattle.[235]

The biochemical steps in stimulation of progesterone synthesis by LH in luteal cells, the details of which are

available in Chapter 23 and have been reviewed elsewhere,[229] include activation of protein kinase A, transport of cholesterol through the cytoplasm, and transport of cholesterol from the outer to inner mitochondrial membrane. Phosphorylation of steroidogenic acute regulatory protein (StAR) by protein kinase A, which appears to be the primary effect of the protein kinase pathway on activity of the StAR protein, enhances cholesterol transport. Large luteal cells had sixfold more StAR than small luteal cells in ovine corpora lutea, and concentrations of StAR were correlated with serum concentrations of progesterone throughout the estrous cycle. Similarly, endozepine, another protein involved in transport of cholesterol across the mitochondrial membrane, was 2.4-fold greater in large cells, leading those authors to propose that these proteins played a constitutive role in large luteal cells, which, as mentioned in this chapter, secrete most of the progesterone but are unresponsive to LH.[236]

In luteal cells from ewes on days 5, 10, and 15 of the cycle, secretion of progesterone in vitro was positively correlated with expression of mRNA for the gap junctional protein, connexin 43 (Cx43), and rates of observed gap junctional communication.[237] When Cx43 mRNA expression was decreased by Cx43 gene-target small interfering RNA, progesterone secretion by cells from days 5 and 10 was decreased as well. These authors thus suggested that intercellular communication may play some role in luteal secretion of progesterone and raised the possibility that findings from separated large or small luteal cells may not fully reflect the factors involved in the function of an intact corpus luteum.[237]

LUTEAL REGRESSION[1,238]

The major new findings in the ewe since our previous chapter include (1) the effects of progesterone via receptors in membranes of the endoplasmic reticulum of steroidogenic luteal cells and the plasma membranes of lymphocytes, (2) the elucidation of an intraluteal role of oxytocin in the process of luteal regression, and (3) the controversial evidence that interferon tau produced by the conceptus can exit the uterus and act directly on the corpus luteum to upregulate genes and prevent luteal regression, as a part of maternal recognition of pregnancy. Rather than reiterate the details reviewed in the previous edition, we will briefly review that information and concentrate on work since 2004 in the current discussion.

Luteolysis is defined as structural demise of the corpus luteum, which is preceded by loss of the capacity to synthesize and secrete progesterone. Natural luteolysis is dependent upon secretion of a uterine luteolytic factor, $PGF_{2\alpha}$, that acts locally, at least in part, because unilateral hysterectomy maintained luteal function on the ipsilateral, but not contralateral, ovary. It was postulated that $PGF_{2\alpha}$ enters the ovarian artery from the utero-ovarian vein via a countercurrent exchange mechanism that allowed $PGF_{2\alpha}$ to reach the ovary without entering the pulmonary circulation, where it would be enzymatically inactivated in the lungs.[1] However, Bonnin et al.[239] later presented strong evidence for a re-interpretation that the process involves both rapid systemic transport (12% of $PGF_{2\alpha}$ was not oxidized by the lungs) and a slower local diffusion pathway that utilizes the utero-ovarian veins and lymph vessels. Luteal-phase progesterone is a major determinant of the timing of uterine secretion of $PGF_{2\alpha}$, with low concentrations of estradiol playing a permissive role in both that secretion and the luteolytic response.[1] Specifically, increased secretion of $PGF_{2\alpha}$ on days 11 through 14 is programmed by rising concentrations of progesterone early in the luteal phase; this was demonstrated by the ability of progesterone treatment beginning at estrus to advance secretion of $PGF_{2\alpha}$ by 4 days and shorten the cycle.[44] The fall in progesterone at luteolysis and subsequent follicular-phase rise in estradiol prevent premature release of $PGF_{2\alpha}$ early in the next cycle, thus ensuring a normal-length luteal phase. As was the case with the priming effect of progesterone on estrous behavior, this priming action may reflect progesterone-induced ERα in the uterine endometrium.[240] More recently, Banu et al.[241] characterized a prostaglandin transporter (PGT) in ovine endometrium that is highly expressed between days 14 and 16 of the estrous cycle. They provided evidence that its action within endometrial cells involved protein kinases A and C, epidermal growth factor receptor, ERK1/2, and Jun kinase–stress-activated protein kinase cell-signaling pathways. Intrauterine infusions of a PGT inhibitor twice daily on days 11 through 15 prevented luteolysis, maintaining progesterone and extending interestrous intervals beyond 35 days.

The other new work of interest in regard to uterine secretion patterns in relation to luteolysis induced by $PGF_{2\alpha}$ comes from a series of studies in heifers in the laboratory of O. J. Ginther at Wisconsin.[242,243] Their work has pointed to differences in responses to the episodic secretion of $PGF_{2\alpha}$, mimicking natural luteolysis, in comparison with effects seen with bolus injections that are considered to be overdoses. Large bolus dosages of $PGF_{2\alpha}$ produced initial increases in progesterone secretion in sheep and cattle in several studies cited. However, in agreement with earlier work in the ewe,[244] a secretory-equivalent pattern of doses of $PGF_{2\alpha}$ initially inhibited progesterone secretion, and yet did not induce structural luteolysis. However, it caused a transient reduction in progesterone, which led to an LH pulse during the decline in $PGF_{2\alpha}$, and these events were followed by a rebound in progesterone. This sequence was repeated during the period that Ginther designated as preluteolysis. The transition to luteolysis began from a point at which progesterone was at its original concentration.

Beyond that transition point, as further episodes of secretion of $PGF_{2\alpha}$ occurred, rebounds in progesterone were incomplete, but partial rebounds continued to be associated with increases in blood flow to the corpus luteum. Reduction in progesterone is a key step in structural luteolysis, because it has recently been shown that intraluteal progesterone protects the steroidogenic cells from apoptosis,[245] which will be discussed later in this chapter.

It has been well documented that $PGF_{2\alpha}$ has at least four major actions in the corpus luteum.[228,229] The temporal sequence of these major, and many other, effects of a luteolytic dose of $PGF_{2\alpha}$ is illustrated in Figure 27.12. First, $PGF_{2\alpha}$ stimulates luteal secretion of oxytocin within 2–5 min after an intramuscular injection[246] or within 15 min after beginning infusion into a uterine lymphatic vessel.[253] Second is a rapid decrease in luteal blood flow (detected by 4 h), concomitant with the third action of $PGF_{2\alpha}$, a decrease in the secretion of progesterone (detected as early as 1–2 h and usually by 4 h) due to activation of the protein kinase C (PKC) second messenger pathway. Fourth, $PGF_{2\alpha}$-induced increases in intracellular calcium appear to cause cell death via apoptotic mechanisms and structural luteolysis. Much of the research in the ruminant corpus luteum in recent years has been directed to the mechanism by which $PGF_{2\alpha}$ induces these effects, including the involvement of a fifth action, stimulation of luteal production of the vasoactive factors, prostaglandins, endothelin, angiotensin II, and nitric oxide (NO), as intermediates or collaborators in a luteolytic cascade (reviewed by Skarzynski and Okuda).[254]

In our previous version of this chapter, evidence that changes related to structural luteolysis began even before completion of functional luteolysis was discussed in some detail. Specifically, changes expected to alter the structure of the extracellular matrix, and to be associated with cell death and structural luteolysis, preceded the nadir in progesterone concentrations.[244,247,248] These changes may play a role in functional as well as structural luteolysis, as indicated by the observation that subluteolytic infusion of $PGF_{2\alpha}$ induced precipitous declines in tissue inhibitor of metalloproteinase 1 and 2 (TIMP1 and TIMP2) proteins lasting 8 or 24 h, respectively,[248] and by the decline in mRNA for TIMP2 in corpora lutea during the late luteal phase.[255] In contrast, mRNA in ovine corpora lutea increased for MMP14 on day 4 and was greatest for MMP2 on day 10, associated with increases in gelatinolytic activity.[256] MMP2 and MMP9 were localized primarily in large luteal cells, but MMP2 appeared to be present in endothelial cells on day 15. These authors concluded that remodeling of the extracellular matrix occurs throughout the luteal phase and may "facilitate processes important to luteal development, maintenance and regression".[256]

A further understanding of intraluteal regulation of luteal regression began with the demonstration of membrane receptors for progesterone in the endoplasmic

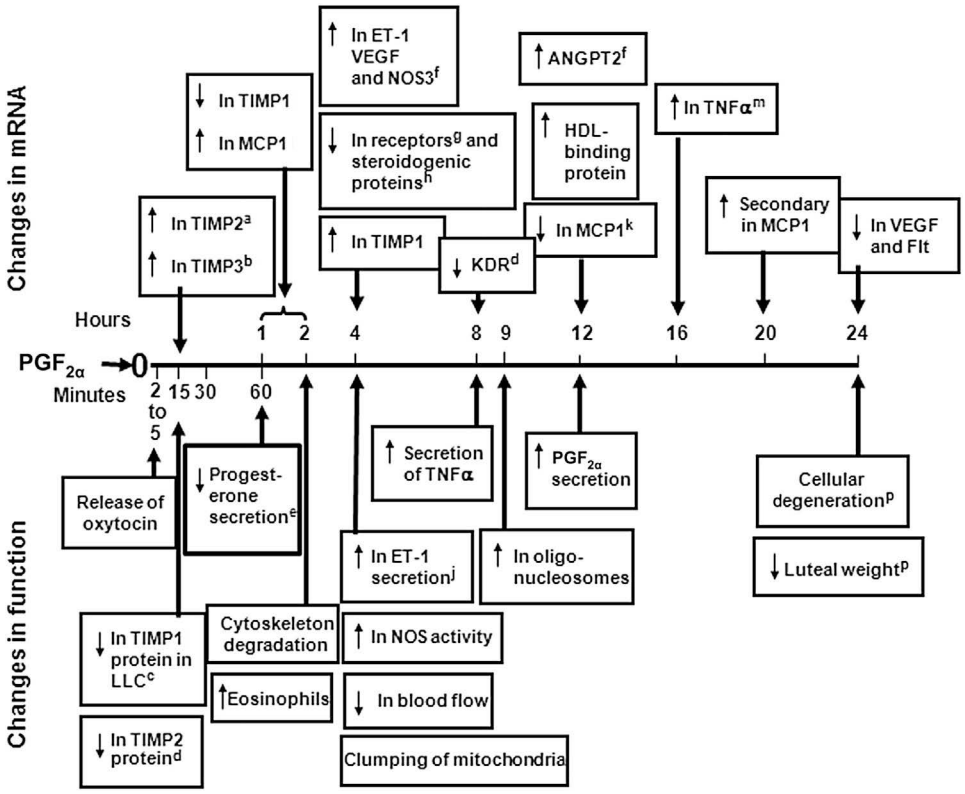

FIGURE 27.12 Time course of intraluteal events in response to a bolus injection of $PGF_{2\alpha}$. Data on messenger RNA are presented above the timeline, and data on functional and structural changes are presented below the timeline. [a]Continues through 48 h. [b]Continues through 6 h. [c]Continues through 8 h. [d]Continues through 24 h. [e]More often detected by 2–4 h because of sampling at same times as for blood flow. [f]Continues through 12 h. [g]Low-density lipoprotein, $PGF_{2\alpha}$, LH, Flt (receptor for VEGF). and Tie-2 (receptor for ANGPT2). [h]StAR, 3βHSD, and P450scc. [i]Continues through 20 h. [k]Continues through 16 h. [m]Occurs in luteal macrophages. [p]Through 36 h and beyond. *Source: Data were adapted from references cited in the text[244,246–252] and References 342,378,384–386,389,402 from the previous edition's version of this chapter.[1] Effects of pulsed $PGF_{2\alpha}$ may vary as discussed in the text based on data in the cow.[242,243]*)

reticulum of both large and small steroidogenic ovine luteal cells.[229,257] The intracellular nature of these receptors was indicated by the lack of effectiveness of a progesterone–BSA conjugate. Localization to the endoplasmic reticulum was demonstrated with fluorescent protein conjugates to the receptor in transfected Chinese hamster ovarian cells. In a 1981 review,[258] Rothchild had summarized evidence that progesterone is an intracrine regulator of luteal function, but it was largely ignored until evidence surfaced for membrane receptors for steroids. This included a report of membrane receptors for progesterone in the rat corpus luteum,[259] and that progesterone was able to oppose functional and structural luteal regression in the absence of classical nuclear progesterone receptors in this species.[260] Membrane receptors also were shown to allow intracellular mobilization of Ca^{++} from the ovine luteal endoplasmic reticulum in response to either progesterone or 17α-hydroxy-progesterone.[257]

Early work[261] showed an ability of oxytocin to increase secretion of $PGF_{2\alpha}$ by the uterus, and the concept of a positive-feedback loop between luteal oxytocin and uterine $PGF_{2\alpha}$ gained wide acceptance.[1] The upregulation of oxytocin receptors in the endometrium was considered key to that loop, with the initial stimulation of secretion of $PGF_{2\alpha}$ by oxytocin from the posterior pituitary. Evidence related to that concept was discussed in our previous review, including some that questioned its importance, such as the indication that increased uterine responsiveness to oxytocin did not precede initial decreases in progesterone secretion and depended upon estrogen being the dominant steroid. In a recent study, an inhibitor of PGT in ovine endometrium, infused into the uterus, blocked oxytocin from inducing pulses of $PGF_{2\alpha}$ on day 15.[241] Considered in relation to the discussion in the previous edition, it remains likely that oxytocin stimulates the final secretion of $PGF_{2\alpha}$ that contributes to structural luteolysis.

A new concept for the role of luteal oxytocin in luteal regression has emerged from the work of Gordon Niswender and colleagues at Colorado State University.[229] The large steroidogenic luteal cells have receptors for $PGF_{2\alpha}$ and produce more prostaglandin in response to the initial stimulation by uterine $PGF_{2\alpha}$. To test whether luteal secretion of $PGF_{2\alpha}$ is required for luteolysis, this group placed implants containing indomethacin in the corpus luteum of ewes on day 11 of the estrous cycle. Serum progesterone declined by day 17 and ewes returned to estrus, but luteal weights on day 18 were heavier in ewes treated with indomethacin than in control ewes. Because peaks of the inactive metabolite of $PGF_{2\alpha}$ (15-keto,13,14-dihydro-$PGF_{2\alpha}$), observed after initiation of luteolysis, did not differ between intact and hysterectomized ewes, the $PGF_{2\alpha}$ secreted in response to treatment was not of uterine origin. In addition to secreting $PGF_{2\alpha}$, the large cells respond to $PGF_{2\alpha}$ by releasing stored oxytocin. The small luteal cells have oxytocin receptors, and the binding of the locally secreted oxytocin elicits increases in PKC and release of intracellular Ca^{++}, which initiates apoptosis of the small cells. Meanwhile, uterine and luteal $PGF_{2\alpha}$ elicits decreases in progesterone and increases in PKC in the large cells, followed by increased intracellular Ca^{++} and apoptosis of those cells.

The evidence that intraluteal regulatory mechanisms are key to life or death of the corpus luteum became increasingly complicated with further studies of ovine luteal cells.[245] As mentioned earlier, the luteoprotective actions of progesterone involve increased intracellular Ca^{++} via the membrane progesterone receptors in the endoplasmic reticulum in both large and small steroidogenic cells.[257] In contrast, $PGF_{2\alpha}$ increased intracellular Ca^{++} to cause apoptosis of large steroidogenic luteal cells, while oxytocin increased intracellular Ca^{++} in causing apoptosis of small steroidogenic cells.[245] Moreover, progesterone blocked these effects of $PGF_{2\alpha}$ and oxytocin on intracellular Ca^{++}. When these results are considered in relation to the protective effect of progesterone against luteolysis[229,245] and the fact that the steroidogenic effect of LH also increases intracellular Ca^{++},[228] it becomes apparent that increases in intracellular Ca^{++} can have a myriad of different effects, depending up the conditions and stimuli.

Another component of luteal regression appears to be the influx of immune cells into the tissue, which is important for structural luteolysis. In our previous review, we emphasized the role of monocyte chemoattractant protein 1 (MCP1, now known as CCL2) in recruiting macrophages in sheep, but most recent work has been in the bovine corpus luteum. Because of evidence for important mechanistic effects of episodic $PGF_{2\alpha}$,[242,243] Atli et al. used two or four episodic low-dose treatments of $PGF_{2\alpha}$ in nonlactating Holstein cows to examine gene expression for factors that activate the immune system.[262] Specifically, the second pulse of $PGF_{2\alpha}$ increased mRNA for interleukins 1B and 8 (IL1B and IL8), FAS, and FAS ligand. Recent reviews provide more detail on the immune cells and cytokines involved in luteal regression, including T lymphocytes of several types, γδ, $CD4^+$, and $CD8^+$.[263,264] Based upon these studies of bovine corpora lutea,[265] $CD8\alpha\alpha^+$ and $\gamma\delta^+CD8\alpha\alpha^+$ T cells that migrate into spaces between steroidogenic cells in the corpus luteum increase during luteal regression. In contrast to the steroidogenic luteal cells, the membrane receptors for progesterone on these lymphocytes are located on the plasma membrane.[266] These membrane receptors appear to account for the ability of progesterone to inhibit luteal cell–induced proliferation of T lymphocytes. The T lymphocytes also have oxytocin receptors[267] and, like small steroidogenic cells, responded to oxytocin with increased intracellular Ca^{++} via the phospholipase

C (PLC)–PKC pathway. Thus, a part of the effect of the intraluteal action of oxytocin in luteal regression may occur by regulation of the resident immune cells.

In the study with dairy cows discussed above, the first pulse of $PGF_{2\alpha}$ increased mRNA for steroidogenic acute regulatory protein and VEGFA, but these mRNAs were decreased by subsequent treatments, which also induced pathways for the production of $PGF_{2\alpha}$. Corpora lutea collected from superovulated ewes were examined at 0, 4, 8, 12, and 24 h after a single luteolytic injection of $PGF_{2\alpha}$ on day 10 of the cycle.[249] They observed similar initial increases in mRNAs for the VEGF and one of its receptors (Flt) at 4 h, decreases in another VEGF receptor (KDR) at 8–24 h, and increases in FGF and angiopoetin 2 at 8 h (Figure 27.12), followed by decreases in the stimulated factors at later times as apoptosis increased and the endothelial component of the vascular bed decreased. In contrast, smooth muscle cell actin remained high during luteal regression, which was considered to indicate a role for pericytes and vascular smooth muscle cells in the process.

Three other vasoactive substances have been shown to increase in ruminant corpora lutea in response to $PGF_{2\alpha}$: endothelin 1 (EDN1),[268] angiotensin II,[249] and NO.[254] Shirasuna et al.[269] observed positive correlations of concentrations of $PGF_{2\alpha}$, EDN1, and oxytocin by microdialysis in bovine corpora lutea during spontaneous luteolysis, but a low relationship to angiotensin II. Oxytocin and $PGF_{2\alpha}$ increased together in ovarian venous plasma in that study. Observations in sheep and cattle indicate that intraluteal secretion of $PGF_{2\alpha}$, oxytocin (both discussed earlier), EDN1, and angiotensin II in response to uterine $PGF_{2\alpha}$ is integral to the luteolytic cascade during the estrous cycle in ruminants.

EDN1 has received detailed study in the ewe; a luteolytic dose of $PGF_{2\alpha}$ stimulated gene expression for EDN1 in ovine corpora lutea,[250] and in a study using microdialysis in vivo,[251] perfusion of $PGF_{2\alpha}$ increased secretion of EDN1 over a period of 12 h (Figure 27.12). EDN1 inhibited basal and LH-stimulated secretion of progesterone in dispersed ovine luteal cells, and the effect was blocked by an antagonist specific for the receptor for endothelin, endothelin receptor type A (EDNRA).[250] The EDNRA antagonist also delayed the decrease in plasma progesterone in response to $PGF_{2\alpha}$. In another study,[268] chronic infusion of the inhibitor into the sheep corpus luteum by an osmotic minipump did not prevent the initial decrease in progesterone in response to $PGF_{2\alpha}$, but restored progesterone to luteal-phase concentrations by 48 h. This result led to the conclusion that the initial action of $PGF_{2\alpha}$ on progesterone secretion was direct, while later stages of luteolysis may be mediated in part by EDN1. In a repeat of that study, the effect was confirmed in three of 12 ewes, and treatment maintained greater expression of mRNA for 3β-hydroxy-steroid dehydrogenase and TIMP1, while it decreased expression of pre-pro-EDN1 and EDNRA.[270] In contrast to the above results, chronic infusion of EDN1 into the ovarian pedicle or intrauterine infusion on days 10 through 18 prevented luteal regression and increased the ratio of PGE_2 to $PGF_{2\alpha}$.[271] Injection of 100 μg EDN1 alone did not regress ovine corpora lutea, but regression was observed in ewes treated with 100 μg EDN1 in combination with a subluteolytic dose of $PGF_{2\alpha}$.[250] Likewise, $PGF_{2\alpha}$ followed by EDN1, simultaneous perfusion of $PGF_{2\alpha}$ and EDN1, two successive perfusions of EDN1, or EDN1 followed by TNFα decreased progesterone secretion and caused a massive release of oxytocin in the study using microdialysis of ovine corpora lutea.[251] TNFα, which is secreted by luteal cells and macrophages, was elevated in regressing ovine corpora lutea after progesterone secretion had decreased[252] (Figure 27.12).

There are different responses of ovine and bovine corpora lutea to NO at various stages of the cycle, and in various in vivo or in vitro conditions, but the authors of a review of this literature concluded that reduced concentrations of NO may facilitate the upregulation of the EDN1 system in older or regressing corpora lutea.[254] Responses of NO appeared to differ in regard to functional and structural regression. For example, activity of endothelial NOS increased at 4 h after treatment with PGF_2 at midcycle.[249] In contrast to reports that NO is luteolytic, uterine infusion of NO donors during days 8–18 of the ovine estrous cycle produced heavier corpora lutea, greater progesterone in the jugular vein, and a greater ratio of PGE_2:$PGF_{2\alpha}$ in the inferior vena cava in ewes that received the NO donor.[272] That interpretation could fit with the conclusion that decreased NO contributed to structural luteal regression.[254]

Overcoming or preventing luteal regression during maternal recognition of pregnancy, although not germane to the estrous cycle per se, provides insight into luteal regression in relation to changes that are prevented. Interferon tau, produced by the trophoblast of the conceptus, is a major signal in the maternal recognition process,[264,273] and evidence for the involvement of prostaglandins E_1 and E_2 in this process has existed since the 1970s (reviewed by Weems et al.[274–276]). It was considered that interferon tau acted in the uterus to alter the ratio of PGEs to $PGF_{2\alpha}$, but evidence has developed that interferon tau may in fact leave the uterus and act directly on the corpus luteum. This possibility initially arose from observations that intrauterine and subcutaneous injections of interferon tau were equally effective in inducing the expression of two proteins in the corpus luteum.[277] Subsequently, it was found that infusion of interferon tau into the uterine vein extended the luteal lifespan in ewes,[278] and a series of studies summarized by Hansen et al.[279] provided evidence that interferon tau is released into the uterine vein and may exert direct

effects on the corpus luteum. However, Lee et al. were unable to detect transport of interferon tau through the utero-ovarian plexus.[280] Instead, they found transport of uterine PGE_2 to the ovary by that route and upregulation of intraluteal biosynthesis of both PGE_2 and its receptors, EP2 and EP4. Moreover, PGE_2 acted through EP2 receptors to increase mRNA for LH receptors, LH receptors, and circulating progesterone.[281] In that same study, the luteolytic action of $PGF_{2\alpha}$ was mediated via prostaglandin F and EP3 receptors. Overall, the mechanisms of maternal recognition remain controversial, but new possibilities for points of disruption of luteal regression have been identified.

FACTORS AFFECTING LUTEAL SENSITIVITY TO $PGF_{2\alpha}$

An interesting research area for several years has been the effort to understand differences in luteal sensitivity to regression by $PGF_{2\alpha}$. These studies have been conducted in cattle, sheep, and pigs, and as discussed in detail in our previous chapter, there appears to be a great deal of commonality among the species. In the ewe, a single injection of $PGF_{2\alpha}$ usually does not cause luteolysis before day 5, but longer acting analogs of $PGF_{2\alpha}$ were effective as early as day 3 of the cycle. Because most studies have been conducted with single bolus injections of $PGF_{2\alpha}$, results will have to be re-evaluated in view of the recent studies using episodic pulses of $PGF_{2\alpha}$,[243] because repeated treatments upregulated luteolytic mechanisms in the early corpus luteum.[235] Luteal sensitivity to $PGF_{2\alpha}$ could not be accounted for by changes in concentrations of receptor for $PGF_{2\alpha}$, but differences were observed between the early and midluteal phases in mRNA for prostaglandin-endoperoxide synthase 2 (PTGS2), CCL2, and TIMP1 protein in response to $PGF_{2\alpha}$.[1] Similarly, mRNA for and activity of prostaglandin dehydrogenase in the corpus luteum decreased later in the cycle, as did mRNA for two inhibitors of PKC.

In bovine corpora lutea, there is greater expression of mRNA for PKC-ε on day 10 than on day 4,[282] and differential expression of several genes associated with signal transduction pathways, including calcium–calmodulin kinase kinase 2.[283] The latter observation led to further studies of Ca^{++} channels[284] that should eventually help to elucidate how the signal transduction pathways in luteolysis induced by $PGF_{2\alpha}$ differ with stage of the estrous cycle. Two recent studies in the pig also may shed light on stage differences in luteal sensitivity to regression by $PGF_{2\alpha}$. First, intraluteal progesterone may play a protective role against $PGF_{2\alpha}$-induced regression, because blocking progesterone synthesis in early corpora lutea allowed treatment with $PGF_{2\alpha}$ to upregulate mRNAs for PTGS2, phospholipase A2, aromatase (CYP19A1), and caspase 3.[285] In a subsequent study, similar effects were found for chemokines (including IL8 and CCL2) and chemokine receptors.[286]

RESYNTHESIS: A MODEL FOR THE OVINE ESTROUS CYCLE

Model for Cycle

In this section, we will use the previous analysis on the control of the individual components to describe how these components interact to produce the ovine estrous cycle. This systems analysis approach as well as space constraints preclude inclusion of some of the detailed mechanisms described in other sections of this chapter. In this synthesis, it is useful to make a distinction between ovarian events controlled by LH from those controlled by FSH because they function largely independently of each other.

The estrous cycle can be viewed as a sequence of causally related endocrine events, with each step in the sequence initiating the subsequent hormonal change. Because the estrous cycle is a recurring chain of events, the description of these events can begin, in theory, at any point in the cycle. However, the preovulatory LH surge is a particularly appropriate starting point because it initiates formation of a new ovarian structure and occurs on what is conventionally taken as day 0 of the ovine estrous cycle.

The LH surge produces ovulation and luteinization of the follicular remnants, and as the corpus luteum develops, progesterone concentrations begin to rise (Figure 27.13). Because the luteotropic actions of LH are permissive in the ewe, the pattern of progesterone throughout the luteal phase (from ovulation to just before luteolysis) reflects internal factors within the corpus luteum and its secretory capacity; it is thus an indirect consequence of the luteinizing actions of the LH surge. The elevated progesterone concentrations during the luteal phase have several major actions that are critical to the estrous cycle. Progesterone primes the centers in the brain, controlling estrous behavior and the GnRH surge so that the follicular-phase estradiol rise induces estrus and a maximal GnRH surge. The early increase in progesterone (days 3–5) inhibits uterine $PGF_{2\alpha}$ secretion, but also programs the uterus to begin episodic secretion of $PGF_{2\alpha}$ 7–8 days later. It also modifies uterine function so that no premature secretion of $PGF_{2\alpha}$ occurs early in the next estrous cycle, ensuring a normal-length luteal phase in that cycle. Interestingly, two of these three priming actions of progesterone may reflect its ability to induce ERα,[195,240] so the ability of progesterone pretreatment to amplify the GnRH surge may induce ERα as well. Finally, progesterone inhibits tonic LH secretion by suppressing GnRH pulse frequency, so that circulating LH concentrations decrease to a nadir at midcycle and remain low until luteolysis. The low concentrations of LH are necessary for follicular waves, and the minimal support for luteal function allows the increase in $PGF_{2\alpha}$ that occurs between

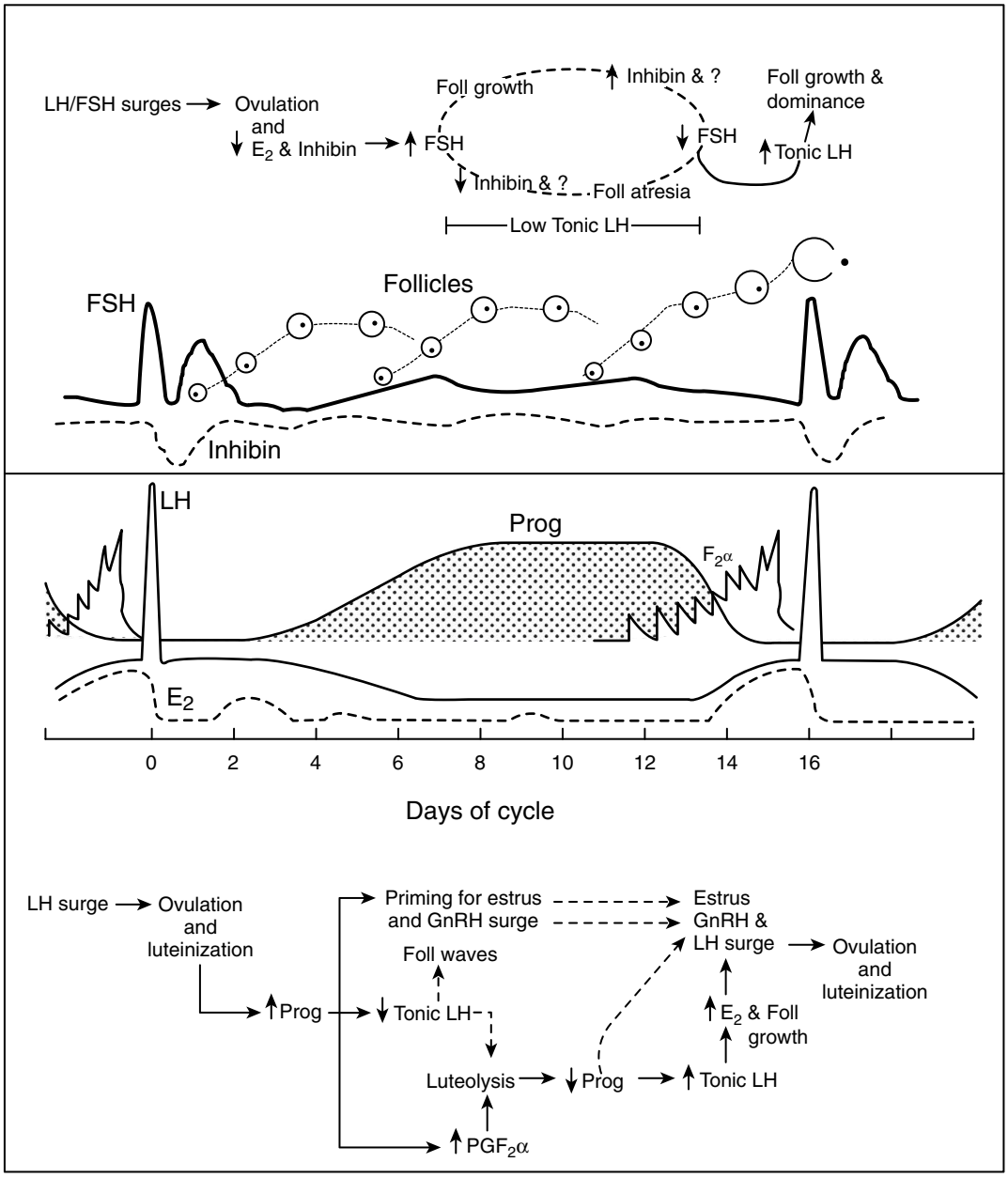

FIGURE 27.13 Model for the two major feedback systems controlling the ovine estrous cycle. The *top half* of the figure presents a schematic depiction of circulating concentrations of FSH and inhibin, the feedback cycle thought to be responsible for waves of follicular development during the luteal phase, and the role of tonic LH secretion in the final stages of follicular growth in the follicular phase. The *bottom half* of the figure depicts a schematic pattern of progesterone (Prog), LH, estradiol (E_2), and $PGF_{2\alpha}$ secretion, and the sequence of causal relationships that determines the timing of major events during the estrous cycle. In the latter, solid arrows represent direct effects, and dashed arrows represent permissive actions. See the text for further details.

days 11 and 13 to induce luteolysis, and consequently progesterone concentrations fall precipitously. The fall in progesterone at luteolysis releases three hypothalamic neural systems from the inhibitory effects of this steroid. It allows the centers controlling the GnRH–LH surge and estrous behavior to respond to estradiol, and allows the GnRH pulse generator to increase in frequency. The resulting rise in tonic LH concentrations helps to maintain follicular growth and stimulates estradiol secretion from the ovary. The sustained follicular-phase elevation in circulating estradiol triggers estrous behavior and the preovulatory GnRH and LH surges, and is also important for programing the uterus for normal $PGF_{2\alpha}$ secretion during the next estrous cycle. The LH surge, in turn, terminates estradiol secretion and induces ovulation and luteinization so that the next cycle begins.

At the same time that these events are occurring, a feedback loop between developing follicles and FSH

secretion helps determine follicular dynamics. The preovulatory LH surge terminates secretion of both estradiol and inhibin from the ovary, and the fall in these two hormones allows FSH concentrations to increase on day 1. This increase in FSH stimulates follicular development and, in the presence of elevated tonic LH, both estradiol and inhibin concentrations increase as a cohort of follicles develops (Figure 27.3). These ovarian hormones in turn inhibit FSH secretion, which eventually causes atresia of these follicles if tonic LH secretion is also low. As follicular function wanes, the fall in their negative-feedback signal allows FSH concentrations to increase again, and a second wave of follicles begins to develop. This temporal sequence is postulated to recur throughout the luteal phase (except that there is little, if any, increase in peripheral estradiol concentrations), producing several waves of follicular development. It should be noted, however, that the limited data now available do not support a role for inhibin in controlling the changes in FSH secretion associated with later follicular waves, so that either an unidentified follicular hormone or ovarian-independent fluctuations in FSH secretion may be critical for some of the follicular waves during the luteal phase. Regardless of the signals involved, the characteristics of the feedback loop between FSH and follicular hormones, and the low concentrations of LH, prevent the development of strong follicular dominance in the ewe. This, in turn, ensures that the ovaries always contain follicles ready to begin the final phase of preovulatory development if luteolysis occurs, which accounts for the short duration of the follicular phase. When luteolysis occurs, the resulting increasing frequency of LH pulses maintains follicular growth in the face of falling FSH concentrations and promotes follicular dominance within that wave so that, in most breeds, only one to two ova are ovulated.

The feedback loop between follicles and FSH secretion functions in parallel with, and relatively independent of, the progesterone feedback loops controlling luteolysis and the events of the follicular phase. Consequently, changes in FSH concentrations play little role in timing the events of the estrous cycle. It is thus not surprising that changes in ovulation rate have little influence on the control of ovarian cycles; estrous cycles in multiple ovulators are very similar to those in ewes ovulating a single ovum.

One important characteristic of this model is the major role of ovarian steroids in control of the estrous cycle. Perhaps the three critical events timing the estrous cycle are luteolysis, estrous behavior, and ovulation–luteinization, and the occurrence of all three is ultimately controlled by ovarian steroids. Progesterone, by ensuring that $PGF_{2\alpha}$ secretion increases late in the luteal phase, controls luteolysis, while the follicular-phase estradiol rise is responsible for estrous behavior and, by triggering the LH surge, ovulation. It is interesting to note that the secretory products of the corpus luteum and preovulatory follicle initiate events leading to the destruction of each structure. The function of both tissues is thus self-limiting, a characteristic that allows for the cyclic nature of ovarian activity. While the preovulatory estradiol rise is critical for events during the follicular phase, it is in turn controlled by progesterone. If progesterone concentrations are high, tonic LH secretion is suppressed, and consequently the estradiol rise cannot occur; if progesterone falls, tonic LH secretion increases and stimulates the preovulatory estradiol increment. One can thus infer that in the ewe, as in the rhesus monkey,[287] a "pelvic clock" controls the estrous cycle, but in the case of sheep, the corpus luteum represents a larger component of this clock than in primates.

Retrospective Historical Analysis

That the corpus luteum plays a primary role in timing the events of the ovine estrous cycle is not a new concept. Indeed, it was first suggested in the 1930s following the experimental observation that lutectomy at mid-cycle resulted in estrous behavior and ovulation within 2–3 days,[3] and a critical role for progesterone was incorporated into the first model for control of estrus and ovulation in the ewe.[8] This model, developed by Robertson prior to the general availability of radioimmunoassays, accurately predicted most of the critical events leading from the demise of the corpus luteum to the induction of ovulation[8]; its only inaccurate prediction was that an increase in FSH was the stimulus for the preovulatory estradiol rise. The importance of progesterone to the ovine estrous cycle was further confirmed by the first measurements of circulating hormonal concentrations throughout the cycle, so that Hansel and Echternkamp concluded in their 1972 review[288] that "the dominant event in the cycle…seems to be the rapid regression of the corpus luteum at the appropriate time." The emphasis on the importance of luteolysis to the control of the estrous cycle sparked interest in the mechanisms controlling luteal lifespan and led to the discovery of the luteolytic effects of $PGF_{2\alpha}$.[289] This discovery, in turn, enabled Goding and associates to develop a model encompassing the complete estrous cycle,[290,291] a model that set the groundwork for many of our current ideas. This model emphasized the role of estradiol, so it was not until the discovery of the negative-feedback actions of progesterone and the importance of these actions to control of the timing of follicular-phase events[13,14] that investigators came back to including a major role for progesterone in models for the estrous cycle.[292,293] This, in turn, led to the model presented in the first edition of *The Physiology of Reproduction*, which we then updated in the third edition to include new information on FSH and follicular

dynamics. Although many important mechanistic aspects of the feedback relationships controlling events during the ovine cycle have been highlighted in the current chapter, the basic relationships between systems have not changed, so the model presented in Figure 27.13 has not been modified from the previous edition.

There is one other important characteristic of this model that is relevant to the control of seasonal reproductive patterns in the ewe. Because the estrous cycle is a sequence of hormonal events, with each step depending on the previous one, disruption of a single step will prevent all subsequent events and thereby cause anovulation. A good example of this phenomenon is pregnancy. As noted in this chapter, the presence of a conceptus in utero blocks luteolysis, probably by stimulating uterine secretion of the luteotropin PGE_2 and possibly by uterine and ovarian actions of interferon tau. Consequently, progesterone does not fall, tonic LH secretion and estradiol do not increase, and ovulation does not occur. This anovulatory condition will then continue until removal of the embryo and fetus by death or parturition allows progesterone concentrations to fall. Similarly, disruption of a single endocrine event accounts for the anovulatory condition of anestrus, although in this case the blockade occurs at a different step in the chain of events comprising the ovine estrous cycle.

SEASONAL CONTROL OF OVARIAN CYCLICITY IN THE EWE

Seasonal Breeding[68,294]

So far, the model described here has considered only the role of endogenous hormonal signals in the control of the ovine estrous cycle. However, as noted in the introduction, reproductive function in ewes is characterized by a marked seasonal variation in fertility that has been a subject of scientific investigation for almost a century. That the annual reproductive cycle of the ewe is controlled by photoperiod was first proposed by Marshall[295] and verified experimentally by Yeates over 60 years ago.[296] The effects of photoperiod in the ewe reflect a complex interaction between day length (hours of light per day) and an endogenous circannual rhythm.[297] This interaction and other aspects of seasonal breeding in sheep and other mammals are considered in detail elsewhere in this book (see Chapter 34) and in recent reviews on seasonal breeding in the ewe.[68,294] Therefore, we will present an overview of this topic, emphasizing the underlying changes in hypothalamic function and their effects on events within the estrous cycle, and recent advances, which include (1) melatonin effects on deiodination of thyroid hormones, (2) characterization of the neural circuitry inhibiting GnRH in anestrus, and (3) the contributions of RF-amides in general, and kisspeptin specifically, to seasonal changes in fertility.

The key observation that laid the foundation for our understanding of these events was the demonstration of a dramatic seasonal variation in the negative-feedback actions of estradiol (Figure 27.14).[131] During anestrus, estradiol suppresses GnRH and LH pulse frequency and thereby strongly inhibits tonic LH secretion, whereas during the breeding season this steroid cannot inhibit pulse frequency and thus produces only a modest inhibition in mean LH concentrations. There is also a modest decrease in LH pulse frequency that is evident during anestrus in ovariectomized ewes, but the physiological significance of this "steroid-independent" effect of photoperiod is unclear.[1]

How Does the Annual Variation in Estradiol Negative Feedback Account for Seasonal Breeding?

As noted in the section Resynthesis: A Model for the Ovine Estrous Cycle, because the estrous cycle is a chain

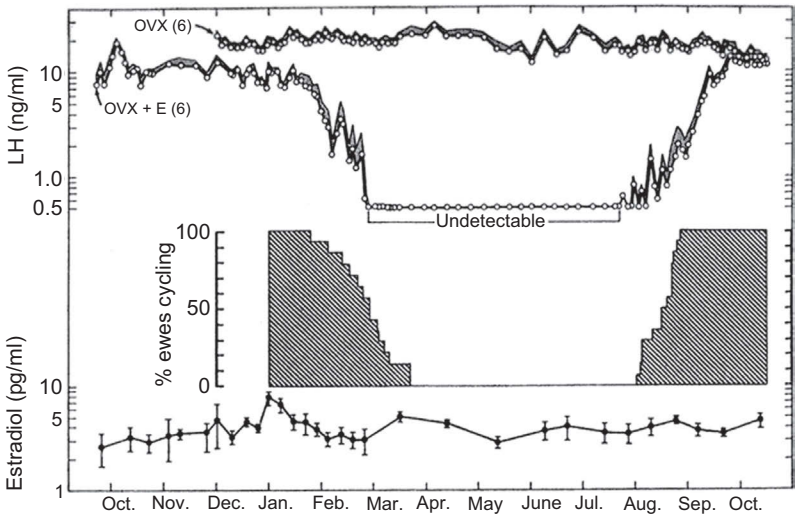

FIGURE 27.14 Seasonal changes in response to estradiol negative feedback. *Top graph*: Mean ± standard error of the mean (shaded area) LH concentration throughout the year in ovariectomized (OVX) or estradiol-treated ovariectomized (OVX + E) ewes. *Bottom graph*: Estradiol was administered with silastic capsules that produced relatively constant serum estradiol concentrations throughout the year. Histogram in the *middle graph* indicates the breeding and anestrous seasons in a separate group of 14 ovary-intact ewes. *Source: Reproduced from Legan et al.[131] (Copyright 1995, The Endocrine Society).*

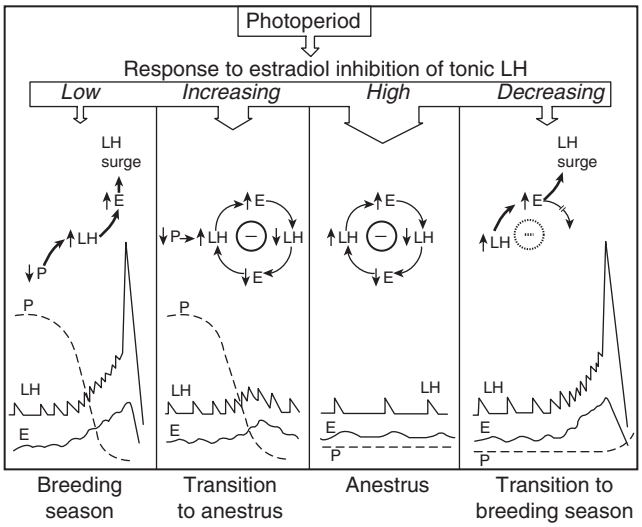

FIGURE 27.15 Model for the control of seasonal breeding in the ewe. Left to right: endocrine events: (a) during the follicular phase in the breeding season; (b) at luteolysis during the transition to anestrus; (c) throughout anestrus; and (d) during the first follicular phase at the transition to the breeding season. See the text for further details. *Source: Reproduced from Karsch et al.*[298]

of causally related events (Figure 27.13), disruption of any step will break the chain and produce anovulation. In terms of seasonal breeding, the critical step is the follicular-phase increase in tonic LH secretion or, more specifically, LH pulse frequency, which drives the preovulatory estradiol rise.[298] During the breeding season, progesterone is the primary inhibitor of GnRH pulse frequency. Hence, when progesterone concentrations fall at luteolysis, GnRH and LH pulse frequencies increase, and the latter stimulates ovarian estradiol secretion; the estradiol rise then triggers the LH surge, and ovulation occurs (Figure 27.15, left panel). At the transition into anestrus, however, the inhibitory neural systems activated by long-day photoperiods allow estradiol to become the primary negative-feedback steroid controlling GnRH pulse frequency. Consequently, following regression of the last corpus luteum of the breeding season, GnRH and LH pulse frequencies do not increase. Without the increase in tonic LH secretion, there is no preovulatory estradiol rise to induce an LH surge and estrous behavior, and anovulation and anestrus result (Figure 27.15, middle panels). Ewes then remain in anestrus as long as estradiol negative feedback holds LH pulse frequency in check. In the autumn, however, the negative-feedback actions of estradiol wane, and, in the absence of progesterone, pulsatile LH secretion increases and stimulates an estradiol rise, which triggers an LH surge, and the first ovulation of the breeding season occurs (Figure 27.15, right panel).

It should be noted that the mechanism controlling tonic LH secretion is not the only hormonal system to show seasonal variations in sheep. A variety of other seasonally related changes has been observed in anestrous ewes, including a decrease in the ability of estradiol to induce estrous behavior, a slight reduction in ovarian responsiveness to LH, and a marked increase in circulating prolactin concentrations.[1] Although these changes may modulate ovarian function and thereby contribute to the maintenance of anestrus, none is sufficient to account for seasonal breeding in the ewe. In contrast, the photoperiod-controlled alterations in tonic LH secretion can explain both the cessation of ovarian cycles in the spring and their reinitiation in the fall.[298]

Neuroendocrine Mechanisms Mediating Seasonal Change in Response to Estradiol Negative Feedback

The mechanisms by which a change in photoperiod is transformed into an alteration in estradiol negative feedback can be divided into three major steps.[68] First, photoperiodic information is received by the retina, and a neural signal is transmitted via the suprachiasmatic nucleus, paraventricular nucleus, and superior cervical ganglion to the pineal gland. Second, the pineal gland transduces the neural signal into an endocrine signal: the serum and CSF melatonin patterns. The pineal secretes melatonin only at night, so the duration of the daily increment in melatonin concentrations is a hormonal analog of the external photoperiod. Third, the melatonin pattern is translated in the hypothalamus to produce a change in its response to estradiol negative feedback.

The exact mechanisms by which melatonin alters hypothalamic function to produce changes in estradiol negative feedback are still under investigation, and there is growing evidence that it acts at different sites to control the two transitions between breeding and anestrous seasons (Figure 27.16). Work focused initially on the transition into the breeding season, with results strongly implicating an area just rostral to the mammillary bodies (the premammillary region) as the site of melatonin action.[299] More recent work raises the possibility that melatonin acts in the pars tuberalis to control the transition into anestrus.[300] The latter studies grew out of data indicating that thyroid hormones are necessary, albeit permissive, for the transition to anestrus.[294,301] However, the hypothesis that these hormones are permissive was based on peripheral administration of thyroxine (T_4), and several lines of evidence indicate that an increase in the local conversion of T_4 to T_3, the biologically active form of thyroid hormone, is critical for the transition into anestrus. As described in detail in Chapter 34, this model proposes that a long-day melatonin pattern acts in the pars tuberalis to induce synthesis and release of TSH.[300] TSH then acts in adjacent neural tissue to increase synthesis of type II deiodinase, the

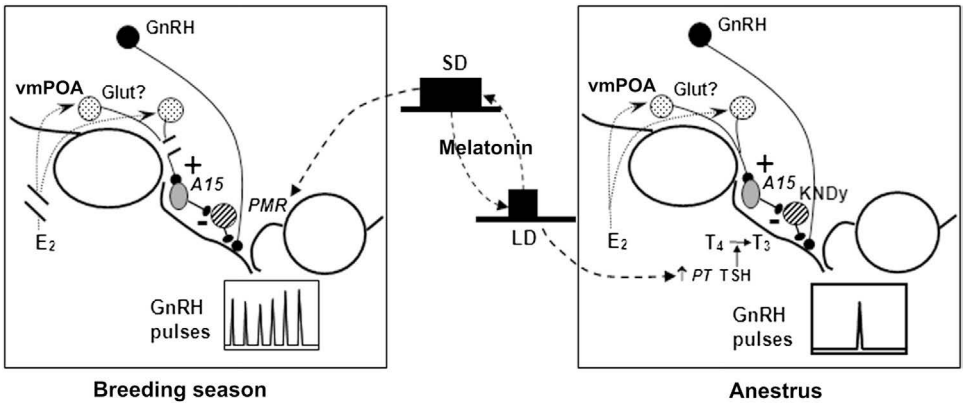

FIGURE 27.16 Model for the seasonal changes in response to estradiol (E_2) negative feedback in the ewe that postulates that an E_2-responsive inhibitory neural system is active only during anestrus. During the breeding season (*left panel*), this system is inactive so that E_2 cannot inhibit GnRH pulse frequency and progesterone is the primary regulator of tonic GnRH secretion. During anestrus (*right panel*), this system is active, allowing E_2 to act on ERα-containing neurons (stippled) in the vmPOA and retrochiasmatic area (that may contain glutamate), which in turn stimulate activity of A15 DA neurons (gray). These DA neurons act in the ARC to inhibit kisspeptin (striped) release from KNDy neurons and thus decrease GnRH and LH pulse frequency. Possible seasonal changes in this circuitry include an increase in responsiveness of the ERα-containing neurons and/or in their ability to stimulate A15 DA neurons. These changes are induced by a long-day (LD) melatonin pattern that increases synthesis and release of TSH in the pars tuberalis (*PT*); TSH then acts locally to increase conversion of T_4 to its active form, T_3, which initiates changes that activate the anestrous neural circuit. The short-day (SD) melatonin pattern, in turn, acts in the premammillary region (PMR) to inactivate this system during the transition to the breeding season.

enzyme that converts T_4 to T_3, and the increasing local T_3 concentrations, in turn, drive hypothalamic changes that shut down GnRH secretion and initiate anestrus. It should be noted that this hypothesis needs to be reconciled with data indicating that T_4 acts in the premammillary region or ventromedial POA, but not in the MBH, to induce anestrus in thyroidectomized ewes.[302] Although further work is thus needed to test this intriguing hypothesis in sheep, these mechanisms may also apply to reproductive transitions induced by long-day photoperiods in hamsters[303] and Japanese quail,[304] indicative of an important evolutionary conservation. Moreover, peripheral administration of T_4 can induce anestrus in thyroidectomized ewes only during a restricted time of the year extending from late breeding season through mid-anestrus,[305] an observation that provides indirect support for the hypothesis that there are important changes in local conversion of T_4 to T_3 during the annual reproductive cycle of sheep.

While the exact mechanisms by which melatonin and/or T_3 control the timing of transitions between breeding and anestrous seasons are yet to be determined, there is now a great deal of information on the neural circuitry within the hypothalamus responsible for the seasonal changes in response to estradiol negative feedback (Figure 27.16). Early work, using pharmacological approaches, led to the hypothesis that an inhibitory DA system suppressed GnRH secretion in anestrus.[1,68] The actual DA neurons involved were identified as the A15 group in the RCh by lesion studies,[306] and subsequent work demonstrated that these neurons are stimulated by estradiol in anestrus, but not in the breeding season, and mediate estradiol negative feedback during anestrus.[1] Thus A15 DA neurons were postulated to play a central role in the hypothalamic changes underlying the seasonal changes in response to estradiol negative feedback. Because A15 neurons do not contain ERα,[52,60] it was inferred that estrogen-responsive afferents are an important part of this circuit, and studies using local administration of estradiol identified such afferents originating from the ventromedial POA (vmPOA)[307] and the ventral RCh.[308,309] These data, together with the observation of an increase in synaptic contacts onto A15 neurons in anestrus,[310] raise the possibility that changes in afferent innervation of these DA neurons are responsible for their selective stimulation by estradiol only in anestrus. More recent work using local administration of receptor agonists and antagonists to the A15 has provided strong evidence that inhibitory GABAergic[311] and stimulatory glutamatergic[312] synapses control A15 DA tone during anestrus, with estradiol inhibiting GABA and stimulating glutamate release at this time of year. Interestingly, there are more glutamatergic-containing close contacts on A15 neurons in anestrus,[312] so that plasticity in estrogen-responsive glutamatergic afferents may account for seasonal changes in estradiol negative feedback. There are no seasonal alterations in GABAergic close contacts onto these DA neurons,[311] but this does not preclude an important role for these inputs in controlling A15 activity. For example, because estradiol inhibits GABAergic tone in anestrus, a loss of ERα expression in these neurons during the breeding season would result in continuous inhibition

of A15 DA activity and thus could account for seasonal changes in response to estradiol.

As with many other aspects of reproductive neuroendocrinology, work during the last decade on seasonal reproduction in sheep has focused on the possible role of kisspeptin and, to a lesser extent, RFRP3. Kisspeptin expression in ARC KNDy neurons was lower in anestrus, largely due to a stronger inhibition by estradiol, and there were parallel changes in kisspeptin-positive contacts onto the more posterior populations of GnRH neurons.[75] No changes were observed in the number of kisspeptin neurons in the POA,[75,313] although there was a small increase in their density in this region during exposure to short-days.[313] These data led to the hypothesis that seasonal changes in kisspeptin are critical to seasonal breeding in the ewe. Subsequent support for this hypothesis came from the observation that exogenous kisspeptin induced ovulation[314] by stimulating tonic LH, and thus estradiol, secretion in anestrus,[315] which demonstrated that inadequate kisspeptin limits fertility at this time of year. The kisspeptin and A15 DA systems may well be coupled based on a report that ARC KNDy neurons contained the DA D2 receptor (with higher expression in anestrus) and that a KISS1R antagonist blocked the ability of an antagonist to the D2 receptor to stimulate LH secretion.[105] These data, together with evidence that A15 DA neurons project posteriorly,[68] led to the hypothesis that the A15 neurons inhibit GnRH pulse frequency in anestrus by limiting kisspeptin release from the ARC KNDy neurons that normally drive GnRH pulses. In summary, the current model for seasonal changes in estradiol negative feedback (Figure 27.16) proposes that in anestrus, estradiol stimulates glutamatergic input in the vmPOA and/or RCh to increase the activity of A15 DA neurons. These neurons, in turn, inhibit the release of kisspeptin from ARC KNDy neurons that are necessary for episodic GnRH secretion, thus inhibiting GnRH pulse frequency. Seasonal changes in this circuitry that prevent its activity during the breeding season most likely occur at multiple levels, and may well include a decrease in ERα in estrogen-responsive afferents,[60] a decrease in stimulatory synaptic input to the A15, decreased responsiveness of ARC KNDy neurons to DA from the A15, and fewer kisspeptin-positive synapses onto GnRH neurons.

Other systems have been implicated as contributing to the seasonal changes in GnRH release, but their physiological significance remains unclear. There is a decrease in synaptic contacts onto POA GnRH perikarya in anestrus,[49] which appears to reflect a shift from stimulatory inputs in the breeding season to inhibitory inputs in anestrus, but these changes are independent of thyroid hormones,[310] so they are unlikely to be critical for seasonal breeding. Changes in RFRP3 may also play a role because the number of RFRP3-immunoreactive cell bodies in the DMH and synapses onto the more rostral GnRH neurons increased in anestrous ewes.[75] There is also one report that RF9, a putative antagonist to the RFRP3 receptors, produced larger increases in LH secretion in anestrous, than in breeding-season, ewes,[112] but these workers were unable to inhibit LH secretion with RFRP3 and concluded that the effects of RF9 were not related to endogenous release of RFRP3. Thus, more work is needed to determine what role, if any, this RF-amide peptide plays in seasonal breeding in the ewe.

Effects of Pheromones and Social Interactions[316–318]

One of the most striking examples of the reproductive impact of social interactions in general, and olfactory input specifically, is the induction of ovulation in anestrous ewes by exposure to rams, which is often referred to as the "male effect". Although this phenomenon has been recognized for more than 60 years and has been put to practical use to induce out-of-season breeding,[319] most of the earlier work has been largely descriptive in nature.[1] However, work during the last decade has provided important new information on underling mechanisms, including the olfactory pathways involved, the importance of learning and memory, and possible changes in hypothalamic function.

It is well established that the exposure of anestrous ewes to a novel ram produces an immediate increase in LH pulse frequency (Figure 27.17), which is followed by follicular growth and a preovulatory LH surge.[1] Ovulations usually occur within 2–3 days of ram introduction, but can sometimes be delayed so that they occur 4–6 days later.[319] These endocrine responses are induced, in part, by androgen-dependent primer pheromones from the head and neck region of the male; the exact chemical nature of these pheromones remains to be determined,[317] but they must be sufficiently complex so that ewes can distinguish between individual rams (as discussed below). It should be noted that estrous females produce similar increases in episodic LH secretion in response to rams, although the functional significance of these effects is less clear.[321]

Olfactory signals from males could theoretically affect GnRH secretion in females via two pathways: (1) the olfactory epithelia that project to the main olfactory bulb, which in turn projects to the cortical amygdala, the piriform cortex, and the entorhinal cortex; or (2) the vomeronasal organ that projects to the accessory olfactory bulb, which in turn projects to the medial amygdala, an area with direct connections to hypothalmic nuclei. The vomeronasal organ and accessory olfactory systems mediate many pheromonal effects on reproduction in rodents,[316] but several lines of evidence implicate the main olfactory system in the responses

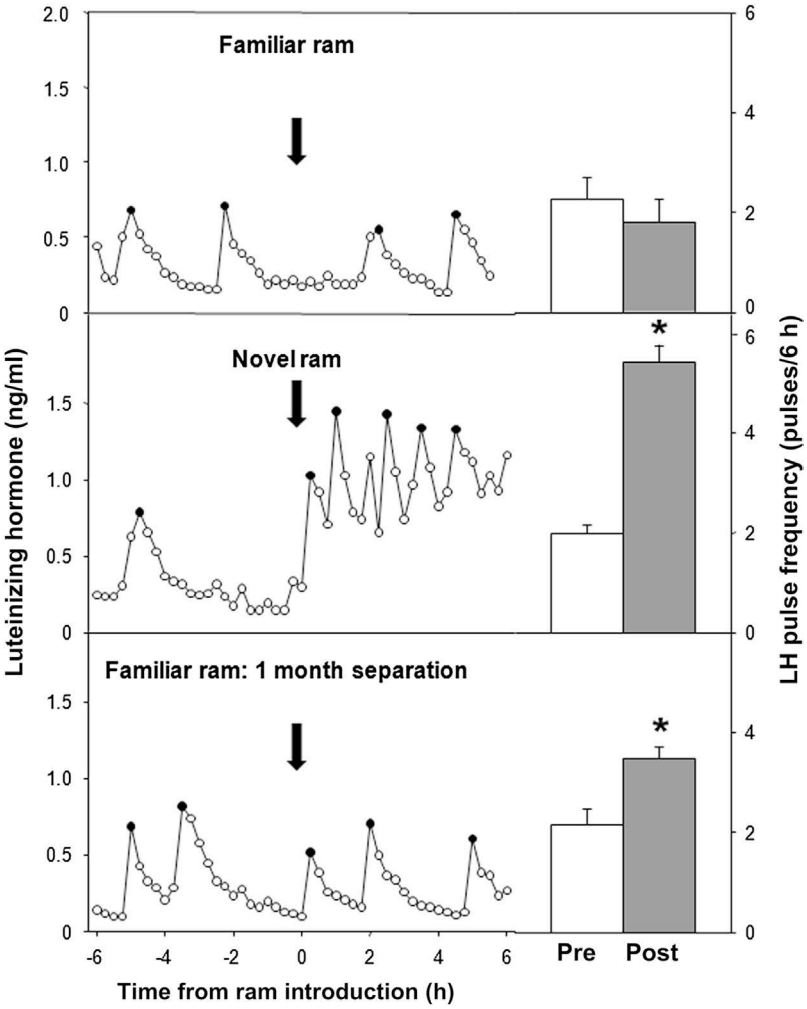

FIGURE 27.17 Effects of introduction of a novel or familiar ram on pulsatile LH secretion in ovary-intact anestrous ewes. Reintroduction of a familiar ram (*top panels*) had no effect on episodic LH secretion, whereas a novel ram (*middle panels*) produces a dramatic increase in LH pulse frequency. Ewes can remember a familiar ram for at least 2 weeks, but by 1 month pheromonal and social signals are able to induce a modest increase in episodic LH secretion (*bottom panels*). Rams introduced at time 0 (arrows). *Source: Adapted from Jorre de St Jorre et al.*[320]

of ewes to male odors. First, male fleece that increased episodic LH secretion also stimulated neural activity (Fos expression) in the main olfactory bulb, the cortical amygdala, and the dentate gyrus of the hippocampus above that induced by female fleece.[322] In contrast, differences in Fos expression between male and female fleece were not seen in neurons of the accessory olfactory system.[322] Second, lesions of the olfactory epithelia blocked the effects of male odor in ewes,[323] but lesions of the vomeronasal organ did not.[324] Finally, inactivation of the cortical amygdala, but not the medial amygdala, prevented male odors from increasing LH secretion in anestrous ewes.[325] It should be noted that the ram provides a much stronger stimulatory signal than that produced by male fleece; rams activated both primary and accessory olfactory systems,[322] and the ram-induced increase in episodic LH secretion could not be blocked by lesions of the olfactory epithelia[323] or inactivation of the cortical amygdala.[325] Moreover, complete disruption of both olfactory systems did not block the ability of rams to induce LH pulses,[323] although the nature of the nonpheromonal signals is still under investigation. Thus rams normally stimulate episodic LH secretion in anestrous ewes via the main olfactory system, with other sensory systems, including the accessory olfactory system and visual input,[326] acting in synergy with the primary olfactory pathway.

It has been proposed that the main olfactory system mediates pheromonal effects in the ewe because it provides olfactory information to cognitive centers and that learning and memory play important roles in this response in sheep.[316] Strong evidence for the involvement of learning was provided by work demonstrating that sexually naïve ewes did not respond to male fleece with an increase in episodic LH secretion.[327] There was a corresponding decrease in activated neural systems in these naïve ewes, with Fos expression increased only in portions of the main olfactory bulb,[328] but not in the amygdala or dentate gyrus, as seen in sexually experienced ewes described above.[322] Apparently naïve ewes have to learn to associate odors with rams because a specific scent (lavender) induced episodic LH secretion if anestrous ewes had been exposed to rams scented with lavender the previous breeding season.[327] An important

role for memory in this process can be inferred from early observations that a novel ram is required to induce this response[1] and has been clearly demonstrated by recent studies.[320] As illustrated in Figure 27.17, a novel ram induced a dramatic increase in LH pulse frequency, whereas a familiar ram had no effect. After exposure, a ewe recognized a ram as "familiar" for at least 2 weeks, but this memory began to fade after a month, although the stimulatory effect at 1 month was not as robust as that of a completely novel ram (Figure 27.17). The mechanisms underlying learning and memory of specific ram odors are unclear, but it is interesting to note that exposure to novel males rapidly stimulated cell proliferation in the dentate gyrus,[329] an area implicated in learning and memory.

While there is thus interesting new information on the afferent pathways and role of learning in the male-induced increase in LH secretion, the changes in hypothalamic function are less clear. Novel males did increase Fos expression in VMN neurons.[322,328] However, male fleece was no more effective than female fleece in this regard,[322] so these neurons are unlikely to be critical for the increase in LH pulses induced by male odors. Novel males were reported to increase Fos expression in a few POA GnRH neurons in one study,[322] but this effect was not observed in a later experiment from this same group.[328] One new relevant observation is that novel males also increased LH pulse frequency during the breeding season.[330] While the impact of this effect on events of the estrous cycle is unclear (see the excellent discussion in Hawken et al.[330]), these data have important mechanistic implications. Previous work had led to the hypothesis that neural systems activated by novel males during anestrus indirectly increase pulsatile LH secretion by disrupting the neural circuitry responsible for estradiol negative feedback,[1,331] but these new data raise the possibility of a more direct effect on the GnRH pulse generator. This possibility is supported by the recent report that exposure of anestrous ewes to rams induced Fos in KNDy neurons and that the increased pulsatile LH secretion in these ewes was blocked by a KISS1R antagonist.[332] It is also consistent with a series of studies in OVX goats in which brief exposure to male odors rapidly (within seconds) induced a single burst of MUA from KNDy neurons.[318,333,334] Interestingly, there was a refractory period after an endogenous MUA burst during which male odors were ineffective,[333] and the burst induced by the male odor delayed the next endogenous MUA burst so that it appeared to reset the pulse generator.[333] These data support a direct interaction of the male stimulus with pulse generation, but caution is needed in applying them to sheep because novel males did not increase LH pulse frequency in OVX ewes, even in anestrus.[331]

CONCLUSION

The ovine estrous cycle is controlled by a complex interaction between cues from the external environment and internal hormonal signals. Environmental factors determine whether or not estrous cycles occur, but are not directly involved in the events of the estrous cycle. Instead, these endocrine events reflect a coordinated hormonal communication among the brain, pituitary, ovaries, and uterus. The primary coordinator of this communication within the ewe is progesterone, which controls the timing of the major endocrine events of the estrous cycle (Figure 27.13). Superimposed on these internal events are two major environmental cues: photoperiods and social interactions with males. Long-day photoperiods, by activating inhibitory neural systems, actively suppress ovarian cycles, but these inhibitory effects of photoperiod can be disrupted by the introduction of pheromones and other signals from the ram.

Although the overall regulation of the ovine estrous cycle involves a large number of hormonal interactions, if one focuses on just the control of ovulation, an important unifying principle emerges. Namely, it is the frequency of pulsatile LH release that determines whether or not ovulation occurs. LH pulse frequency is critical because a relatively fast frequency is required for the sustained estradiol rise that triggers the preovulatory LH surge. Consequently, the activity of the hypothalamic GnRH pulse generator is a pivotal control point under a variety of conditions (Figure 27.18). During the breeding season, estradiol does not inhibit pulse frequency, but progesterone does. Thus, at this time of year, the pattern of progesterone determines the timing of ovulation. When progesterone is high, pulse frequency is low, and anovulation results; when progesterone falls at luteolysis, pulse frequency increases because estradiol can only inhibit pulse amplitude at this time of year, and ovulation occurs within 2–3 days.

During anestrus, estradiol gains the capacity to suppress the hypothalamic pulse generator, so that LH pulse frequency remains low in the absence of progesterone (Figure 27.18). As a result, the animals remain anovulatory as long as estradiol is able to hold episodic LH pulses in check. Exposure of ewes to pheromones from rams may well act via an unknown neural pathway to directly increase GnRH pulse frequency, and hence LH pulse frequency, so that introduction of rams induces ovulation in anestrous ewes (Figure 27.18). Alternatively, the negative-feedback action of estradiol is lost as the effects of long-day photoperiods wane in autumn. Thus, LH pulse frequency increases, which initiates ovulation and the start of the breeding season. With the

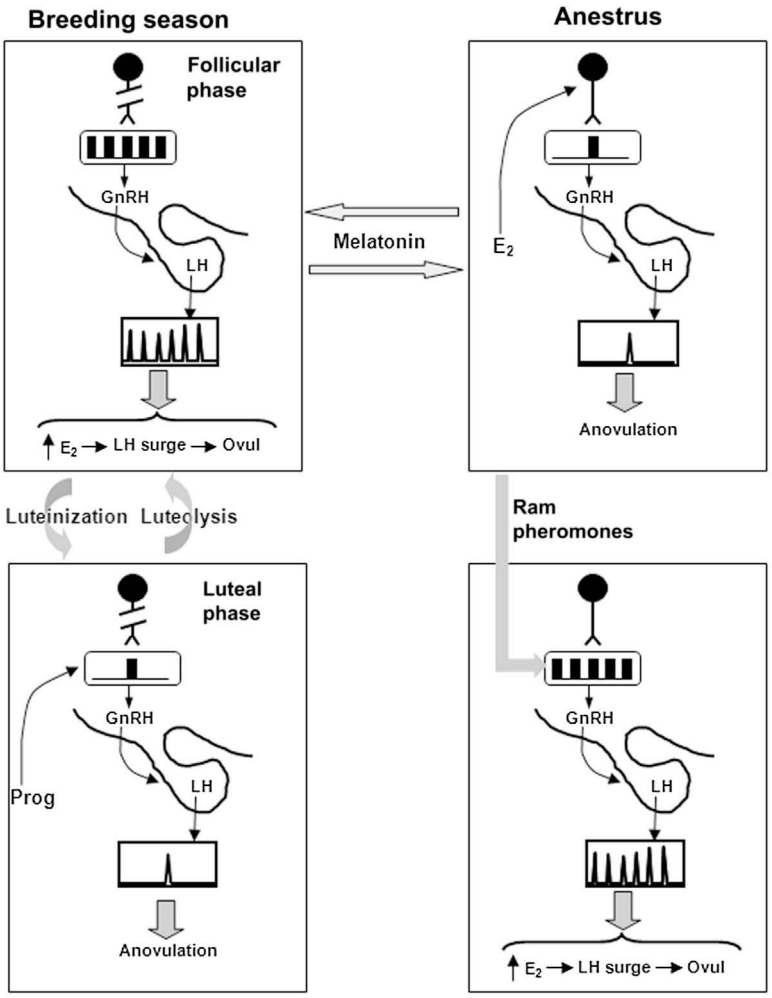

FIGURE 27.18 Control of ovulation in the ewe. The frequency of pulsatile LH secretion, by controlling the preovulatory estradiol (E_2) rise, determines whether or not ovulation occurs. *Left panels*: During the breeding season, progesterone (Prog) is the primary regulator of LH pulse frequency. *Bottom left*: During the luteal phase, Prog inhibits the hypothalamic pulse generator, producing anovulation. *Top left*: Following luteolysis, LH pulse frequency increases and initiates the follicular-phase events leading to ovulation. *Right panels*: During anestrus, the long-day melatonin pattern activates an E_2-sensitive inhibitory neural system. *Top right*: In anestrous ewes isolated from rams, E_2 stimulates the inhibitory neural system, which inhibits GnRH and LH pulse frequency and ensures anovulation. *Bottom right*: Pheromones and social signals from a novel ram activate a neural pathway that directly stimulates the GnRH pulse generator, increasing LH pulse frequency and producing ovulation during anestrus.

return of ovulatory cycles, progesterone concentrations increase, and this steroid resumes control of the hypothalamic pulse generator and hence the occurrence of ovulation.

In updating this chapter, we have identified several unresolved issues that remain to be addressed. In the area of neuroendocrinology, these include (1) testing the hypothesis for the role of KNDy neurons in episodic GnRH secretion; (2) determining the physiological roles, if any, of NKB and RFRP3; and (3) integrating the actions of kisspeptin with those of other neural systems controlling GnRH release. In terms of ovarian function, future work is needed to (1) understand the mechanisms responsible for follicular waves, (2) evaluate the interactions of intraovarian factors in the control of follicular development and estradiol and inhibin secretion, (3) assess the complex interactions of intraluteal factors ($PGF_{2\alpha}$, oxytocin, resident immune cells and cytokines, vasoactive substances, and intracellular Ca^{++}) in the demise of the corpus luteum, and (4) test recently developed hypotheses for the disruption of luteolysis by the embryo. Finally, areas for future work in seasonality include (1) determining the mechanisms by which melatonin and T_3 alter the neural circuitry responsible for seasonal breeding at the transitions into the breeding and anestrous seasons, respectively; and (2) identifying the changes in hypothalamic neural systems by which pheromonal and social cues from the ram increase the activity of the hypothalamic pulse generator.

In conclusion, the basic endocrine interactions among the hypothalamus, pituitary, ovary, and uterus responsible for ovarian function in the ewe are, with a few exceptions, now well understood. Our understanding of these basic mechanisms has led to practical applications in animal husbandry, including the synchronization of estrus for artificial insemination and out-of-season breeding protocols. More recently, significant progress has been made in elucidating mechanistic aspects of these interactions at several levels. Our understanding of critical

neural mechanisms has increased dramatically, as has our knowledge of intraovarian factors controlling follicular and luteal function. This new information provides important conceptual details for, and a richer contextual picture of, the model describing the function of the ovine hypothalamo–pituitary–ovarian axis and may lead to the future development of treatments to improve fertility in domestic livestock.

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CHAPTER

28

Control of the Menstrual Cycle

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INTRODUCTION

Although the regulation of the primate menstrual cycle has been addressed in previous editions of this text, the ovarian events that comprise the menstrual cycle were discussed in isolation from the neuroendocrine control mechanisms that govern these ovarian processes. In this current version of the regulation of the menstrual cycle, we have attempted to integrate the neuroendocrine control mechanisms that govern follicular development, ovulation, and the corpus luteum during the menstrual cycle. We will highlight the importance of the physiological signaling processes that occur between the ovaries and the hypothalamic-pituitary unit that result in a remarkable orchestration of perfectly timed events that culminate in the production and ovulation of a mature oocyte, the formation of the corpus luteum with the concomitant preparation of the uterine endometrium for the implantation of a fertilized oocyte, and the establishment and maintenance of pregnancy. The overall goal of this chapter is to provide a focused review of the extant literature that emphasizes the physiological control mechanisms that govern the timing of the events that occur during the menstrual cycle. The molecular and cellular processes that underpin these events are presented in detail in Chapters 20–23,25.

The history of the ovary as a reproductive and endocrine gland is summarized in contributions by Short and Corner.[1,2,3] The anatomical presence of the ovary has been recognized since the time of Aristotle (384–322 BCE), and the great anatomists of the sixteenth and early seventeenth centuries—Vesalius, Fallopius, and Fabricius—described the vesicular nature of the ovary. In his text *De mulierum organis generationi inservietibus*, de Graaf not only described the ovarian (Graafian) follicle but also surmised that the corpus luteum, previously named by Malphigi, participated in reproduction. De Graaf noted that the presence of a corpus luteum within the ovary was associated with the presence of a fetus in the uterus and that there were a corresponding number of corpora lutea and fetuses in species that bore more than one young. Having deduced the role of the ovarian follicle in the production of the egg (although he concluded that the follicle was the egg itself), de Graaf proposed that the function of the corpus luteum was to squeeze the egg out of the ovary.[3] The correct anatomical relationship between the ovarian follicle and the oocyte was identified by von Baer in 1827.[1]

The endocrine functions of the ovary began to be recognized in the mid-nineteenth century as the result of clinical descriptions on the effects of ovariectomy in humans. In his 1856 text, Ludwig noted that removal of the ovaries in humans led not only to the cessation of menstruation but also to the shrinkage of the uterus.[3] As presented by Corner,[3] the initial thoughts regarding the atrophy of the uterus after ovariectomy were that either the blood or nerve supply to the uterus was disrupted by the surgery. The finding by Knauer in 1896 that castration in guinea pigs followed by grafting ovaries at different sites within the body prevented uterine atrophy indicated that blood-borne chemicals were produced by the ovaries and acted upon the uterus.[3] The description by Allen in 1922 of the cyclic changes in the vagina of rodents and the association of these changes with the presence of mature follicles led to the hypothesis that the uterine development associated with estrus was due to secretions of the maturing follicle, which was subsequently confirmed by the findings that injections of small amounts of follicular fluid into castrated mice and rats led to changes in the vaginal epithelium typical of estrus.[4,5,6] Armed with this sensitive bioassay for the hormone of estrus, Doisy and colleagues purified estrone from human pregnancy urine and porcine follicular fluid.[7,8]

During the early 1900s, research on ovarian secretions progressed on two fronts. In addition to the knowledge that removal of the ovary led to atrophy of

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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the uterus, the role of the corpus luteum in the maintenance of pregnancy was documented when Fraenkel demonstrated that ablation of the corpus luteum in rabbits after mating resulted in the failure of the animals to establish pregnancy.[9,10] The link between the secretions of the corpus luteum and the endometrium was made in 1910 by Ancel and Bouin when they described the histological changes of the rabbit endometrium associated with pregnancy and demonstrated that the removal of corpora lutea prevented the development of the gestational endometrium.[11] In 1928, Corner, after a decade of research on the anatomy and physiology of the corpus luteum, invited Allen, a medical student skilled in organic chemistry, to take a year of sabbatical from his studies to work with him on the corpus luteum. This collaboration led to the isolation of a progestational agent from pig corpora lutea and the demonstration that this material maintained pregnancy in castrated rabbits.[12,13] Research on the chemical nature of the progestational hormone was continued by Allen and others, and progesterone was obtained in crystalline form in 1934.[14,15]

In addition to producing steroid hormones, the ovary was shown to produce a number of peptide hormones including relaxin, oxytocin, and a number of growth factors (see elsewhere in text and Chapters 21 and 23) as well as the peptide hormones inhibins and activins[16] that participate in both the regulation of follicle-stimulating hormone (FSH) secretion in a species-dependent manner and the local (paracrine and autocrine) control of ovarian function (as described later).

The first half of the twentieth century was also the period in which the role of the pituitary gland in controlling ovarian function was defined. As elegantly chronicled by Greep,[17] these years were a period of intense efforts to identify the hypophysial hormones responsible for follicle growth and luteinization. The clinical findings that disorders of the pituitary gland in humans were sometimes associated with sexual immaturity suggested that the pituitary secreted a gonad-stimulating substance, observations that were confirmed when Smith demonstrated that the effects of hypophysectomy on the reproductive organs could be counteracted by extracts of pituitary tissue.[18] In the 1920s, Ascheim and Zondek extracted urine from pregnant and postmenopausal women and identified the presence of two distinct gonadotropic activities: urine from postmenopausal women stimulated follicular development, whereas urine from pregnant women stimulated luteinization.[19,20] Isolation and biological characterization of two distinct pituitary gonadotropic hormones, FSH and luteinizing hormone (LH), was accomplished in the mid-1940s.[21]

The first direct evidence for adenohypophysial control of ovarian function in primates was the independent studies of Allen in 1928[22] and Hartman in 1930[23] that demonstrated that treatment of immature and adult monkeys with pituitary extracts resulted in stimulation of ovarian function. Thereafter, investigations by others demonstrated that follicular development in monkeys could be initiated by pituitary extracts but not by extracts obtained from urine of pregnant women.[24–26] The proof of two distinct anterior pituitary gonadotropic hormones in the 1940s led to rational approaches in the stimulation of ovulation in primates, and the work of van Wagenen and Simpson in the early 1950s demonstrated that follicular growth could be stimulated in monkeys with purified preparations of macaque gonadotropins.[27] Knobil et al.[28] were the first to report that follicular development and ovulation could be elicited in hypophysectomized monkeys with purified gonadotropins, findings that were subsequently confirmed in humans.[29,30]

The notion that the central nervous system (CNS) regulates gonadal function emerged at the turn of the twentieth century, largely as the result of the recognition by Marshall that many environmental cues (termed exteroceptive factors by Marshall) such as food availability, photoperiod, and temperature had a profound impact on reproduction.[31] In humans, the recognition by clinicians that menstrual cycles can be altered by environmental and social stresses, such as those associated with new jobs and failed relationships, also contributed to the idea that the CNS was an important component of the control system regulating ovarian function. Although it was apparent from the early studies of castration and gonad transplantation in cockerels by Bethold in the mid-nineteenth century that the peripheral nervous system was not critical for gonadal function, definitive evidence that the pituitary was the link between the CNS and gonad did not emerge until the ovary stimulating properties of the pituitary were recognized in the late 1920s.[18] The finding that electrical stimulation of the hypothalamus, but not of the pituitary, caused the release of gonadotropins directed attention on this region of the forebrain by those investigating the CNS control of ovarian function.[32,33] The idea that the CNS control of the gonadotropins, and other anterior pituitary hormones, was likely mediated by the liberation of humoral substances released from the hypothalamus into the primary plexus of the hypophysial portal circulation in the median eminence was formulated by Harris in the 1940s.[34] In a key experiment, Harris and Jacobson[35] found that when pituitary glands where placed in the subarachnoid space beneath the median eminence of hypophysectomized female rats, estrous cyclicity was observed in the recipient animals. This did not occur when the donor pituitaries were transplanted to a site distant from the median eminence and hypophysial portal circulation, although the transplanted pituitaries were well vascularized in both locations; it was concluded that the hypophysial

portal blood supply must have a specific property that is capable of activating pituitary gonadotropin secretion.[35] Harris's hypothesis was finally confirmed beyond doubt by the near simultaneous isolation and characterization of a hypothalamic gonadotropin-releasing peptide, now known as gonadotropin-releasing hormone or GnRH (aka LRF, luteinizing hormone releasing factor or LHRH, luteinizing hormone releasing hormone), by the laboratories of Schally and Guilleman in 1971.[36,37] That GnRH was released from the hypothalamus into the portal circulation in an intermittent mode was inferred by Knobil and his colleagues in 1969 from the moment-to-moment changes ("pulses") they observed in LH concentrations in the peripheral circulation of ovariectomized monkeys.[38] Although the critical importance of such a pulsatile pattern of hypothalamic GnRH release in sustaining gonadotropin secretion was established by the same laboratory in 1978,[39] it wasn't until more than a decade later that the intermittent release of GnRH into the hypophysial portal circulation was demonstrated empirically by Clarke and Cummins.[40]

The finding that selective lesions of the arcuate nucleus (a group of neurons immediately dorsal to the median eminence) in ovariectomized monkeys was associated with loss of LH and FSH secretion[41] led Knobil and his colleagues to conclude that the arcuate nucleus was the likely hypothalamic site where the signal for pulsatile GnRH release originated, thus spawning the concept of the hypothalamic GnRH pulse generator. Most recently, findings from human genetics that loss of function mutations of a G-protein coupled receptor, GPR54 (aka, kisspeptin receptor—KISS1R), are associated with hypogonadotropic hypogonadism and absent or delayed puberty[42] have led to an explosion of studies demonstrating that the ligand to the receptor, kisspeptin, which is expressed in neurons in the arcuate nucleus, is of fundamental importance in driving pulsatile GnRH release.[43]

That ovarian hormones were involved in a "push–pull" servo system with gonadal factors serving as circulating inhibitory signals to pituitary gonadotropin secretion emerged from studies rodents by Moore and Price in 1932.[44] That estradiol also exerted a stimulatory or positive feedback action on gonadotropin secretion, triggering the ovulatory LH and FSH discharge at the midpoint of the menstrual cycle, was later demonstrated in the human and monkey female.[45,46]

OVERVIEW OF THE MENSTRUAL CYCLE

Pattern of Gonadotropins, Ovarian Steroids and Inhibin Levels during the Menstrual Cycle

The ovarian cycle may be divided into three stages: the follicular phase, ovulation, and the luteal phase. Figure 28.1 illustrates the circulating concentrations of FSH and LH (top panel), estradiol and progesterone (center panel), and inhibin A and inhibin B (lower panel) throughout the human menstrual cycle.[47] Because of differences in the duration of the follicular phase, values are standardized to the day of the mid-cycle LH surge (Day 0).

The follicular phase commences upon the initiation of menstrual bleeding from the previous cycle and lasts until the mid-cycle LH surge. The early part of the follicular phase is characterized by elevated concentrations of FSH and low concentrations of LH. Concentrations of the ovarian hormones estradiol and progesterone are also low. Inhibin A concentrations are low while inhibin B concentrations rise, lagging slightly behind those of FSH. Approximately 7–10 days before the mid-cycle gonadotropin surge, serum estradiol and inhibin A concentrations begin to rise and continue to increase thereafter. Coincident with the progressive rise in estradiol and inhibin A concentrations is a decline in FSH concentrations, while LH concentrations are maintained or continue to increase gradually. The rise in estradiol and

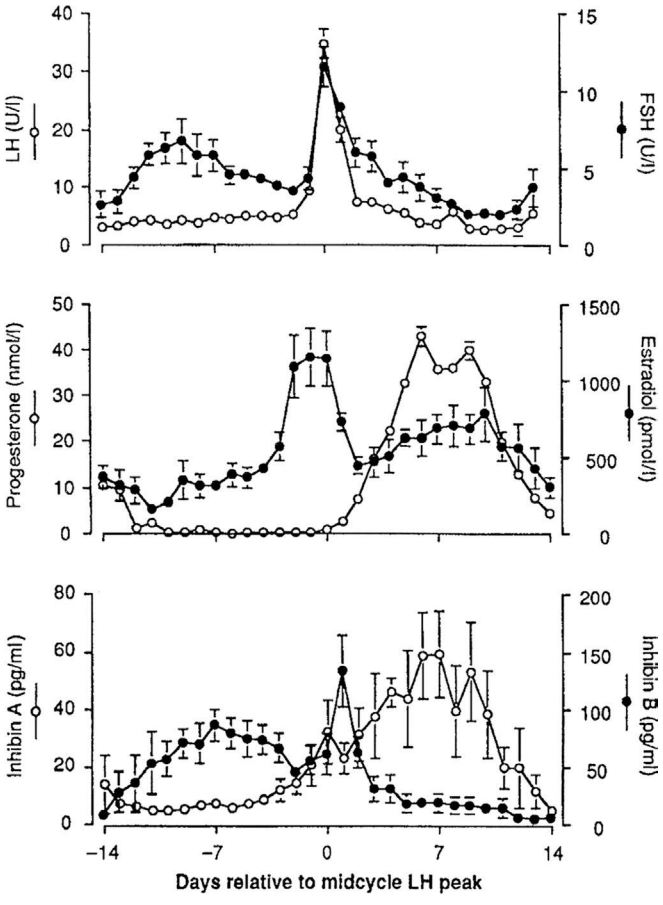

FIGURE 28.1 Profiles of the concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, and inhibins throughout the human menstrual cycle. *Source: Redrawn from Ref. 47 with permission, Copyright 1996, The Endocrine Society.*

inhibin A production during the mid-follicular phase onwards reflects the emergence of the follicle destined to ovulate. At this time, asymmetrical estradiol secretion, as assessed by direct sampling of ovarian venous blood, becomes evident, and mechanical destruction of the largest visible follicle within the ovaries delays ovulation by the two weeks required for the maturation of a new preovulatory follicle.[48,49] The follicular phase, in addition to producing an oocyte that is capable of being fertilized and establishing a pregnancy, is also a period during which the uterine endometrium is stimulated to proliferate under the influence of the estradiol that is produced by the developing follicle.

The follicular phase ends with the abrupt elevation of serum LH (and FSH) levels that causes the rupture of the follicle and the release of its mature oocyte (ovulation). As part of the dramatic structural and functional changes that accompany ovulation, there is a marked fall in estradiol and inhibin B production from the follicle. Thereafter, blood levels of progesterone begin to rise, heralding the transformation of the ovulated follicle into a corpus luteum and the beginning of the luteal phase of the menstrual cycle. Inhibin A concentrations continue their late follicular phase rise and then fall in parallel with those of progesterone. The primate corpus luteum also secretes estradiol so concentrations of this steroid parallel those of progesterone during the luteal phase. The progesterone that is produced by the corpus luteum transforms the proliferative endometrium to a secretory one in anticipation of the implantation of a fertilized oocyte. In the absence of pregnancy, the corpus luteum has a 14–16 days lifespan following which production of progesterone and other luteal hormones ceases, menstruation commences, and another ovarian cycle starts anew. However, in the event that successful fertilization and implantation occurs, the lifespan of the corpus luteum and its resultant production of progesterone are prolonged until the placenta develops the capacity to produce sufficient amounts of progesterone to maintain the established pregnancy.

The Concept of a Pelvic Clock

It is apparent in Figure 28.1 that precise timing and communication is required between the ovary and the pituitary gland to ensure that the mid-cycle surge in gonadotropins occurs at the point in time of the menstrual cycle when an ovarian follicle that is capable of ovulating and forming a corpus luteum is present within the ovary. The classical studies of Knobil and his colleagues in rhesus monkeys investigated the effects of ovariectomy plus replacement of steroid hormones on the generation of an LH surge. These studies convincingly demonstrated that the ovarian hormone estradiol was the principal regulator of the timing of the LH surge and that peripheral estradiol concentrations must be elevated to ~1000 pmol/l (275 pg/ml) for 24–36 h in order for an LH surge to occur.[50] As can be seen in Figure 28.1, in women this concentration of estradiol is seen during the late follicular phase and immediately precedes the spontaneous LH surge. On the basis of these observations, Knobil concluded that the clock that times the onset of the LH surge, and therefore ovulation, lies within the pelvis and its zeitgeber (synchronizer) is the ovary through its production of estradiol. As will be discussed later, not only is estradiol responsible for the timing of the LH surge and ovulation but it is also involved in the process by which a single follicle is selected to mature during the follicular phase of the menstrual cycle.

The Hypothalamic GnRH Pulse Generator

In the primate hypothalamus there are some 1000 to 2000 diffusely distributed GnRH neurons (see Chapter 11), many of which send their projections to the median eminence where they synchronously and intermittently discharge their peptide into the primary plexus of the hypophysial portal circulation, thereby providing the pituitary gonadotropes with the pulsatile stimulation that is obligatory for maintaining gonadotropin secretion.[39] While routine assessment of GnRH concentrations in portal blood is not feasible in primates, studies of the ewe indicate that discharges ("pulses") of GnRH into the hypophysial portal circulation are robustly correlated with episodes ("pulses") of LH secretion from the pituitary.[40,51] Moreover, in several species including the rhesus monkey volleys of multiunit electrical activity (MUA) that are tightly coupled to pulses of LH secretion (Figure 28.2) can be recorded in the medial basal hypothalamus (MBH),[52,53] and it is thus generally recognized that hypothalamic MUA provides a robust correlate of GnRH pulse generator activity.[54] In the absence of ovarian negative feedback on gonadotropin secretion, the primate GnRH pulse generator operates at a frequency of approximately 1 pulse/h.[38,52]

The early finding by Knobil's laboratory in the female rhesus monkey that pulsatile LH secretion was maintained after all neural inputs to the MBH had been surgically interrupted using a bayonet-shaped "Halasz" knife[55] suggested that the neurobiological network necessary for pulsatile GnRH secretion was resident in this basal aspect of the hypothalamus that lies between the optic chiasm and mammillary bodies. The later finding by the same laboratory that discrete lesions of the arcuate nucleus in the MBH of ovariectomized monkeys abolished gonadotropin secretion without compromising the pituitary's response to GnRH, while much larger lesions in this region of the hypothalamus that spared the arcuate nucleus had little impact on LH and FSH secretion,[41] led Knobil to propose that the origin of GnRH

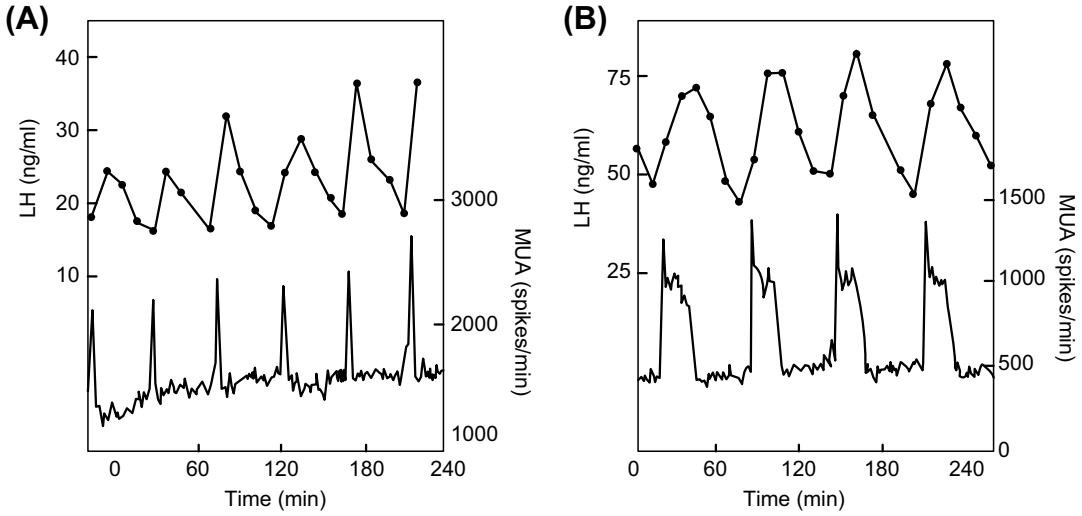

FIGURE 28.2 Multiunit electrical activity (MUA) recorded from the mediobasal hypothalamus in a normal monkey on day 5 of the follicular phase of the menstrual cycle (A) and in an ovariectomized animal (B). Note the association of the MUA volleys with the initiation of luteinizing hormone (LH) pulses and the shorter duration of the volleys in the presence of the ovary. *Source: Reproduced from Ref. 52 with permission, Copyright 1991, S. Karger AG, Basel.*

pulse generation in the primate hypothalamus resides within the arcuate nucleus.[56] Since the vast majority of GnRH neurons in the monkey MBH are found lateral to the midline arcuate nucleus surrounding the base of the third ventricle,[57] they would have likely remained intact in the arcuate lesioned animals (Figure 28.3). This would suggest that pulse generation is actively imposed upon the network of GnRH neurons rather than reflecting an intrinsic property of the GnRH neurons per se. The concept of the GnRH pulse generator remained that of a black box for essentially 30 years. With the landmark discovery in 2003 that loss of function mutations of the kisspeptin receptor (KISS1R, aka GPR54) in man were associated with hypogonadotropic hypogonadism and delayed or absent puberty,[42,43] a neurobiological basis to account for GnRH pulse generation by the arcuate nucleus has begun to emerge.

Within the MBH in primate and nonprimate species, expression of kisspeptin at both the mRNA and protein levels is restricted primarily to neurons in the arcuate nucleus[57–59] (see also Chapter 11). Kisspeptin fibers project throughout the arcuate nucleus and to the median eminence where they mingle intimately with GnRH fibers targeting the primary plexus of the hypophysial portal circulation[57] (Figure 28.3). That kisspeptin provides an attractive candidate to account for the intermittent signal relaying the output of the GnRH pulse generator to the GnRH neuronal network is suggested by the following observations. First, GnRH neurons in nonprimate species have been shown to express KISS1R,[60,61] and application of microdialysis to assess peptide release in the median eminence of the female monkey suggests that both GnRH and kisspeptin release in this region of the MBH is pulsatile and that the discharge of these neuropeptides can occur synchronously.[62] Second, concomitant application of a KISS1R antagonist in the dialysate perfusing the monkey median eminence results in a suppression of pulsatile GnRH release.[63] Third, single IV bolus challenges with kisspeptin throughout the human menstrual cycle invariably evoke an LH discharge,[64] and in the juvenile monkey where spontaneous pulsatile GnRH and LH secretion is minimal, chronic repetitive IV infusions of kisspeptin at 1 pulse/h (the open loop frequency of the GnRH pulse generator in the adult) elicits, as reflected by LH release, a sustained train of GnRH pulses comparable to that generated spontaneously by the hypothalamus of the adult.[65] Fourth, continuous infusions of kisspeptin (kisspeptin-10) at various low doses (0.3–4.0 μg/h/kg) in the latter nonhuman primate model failed to stimulate any parameter of LH secretion (Ramaswamy and Plant, unpublished observations), while high dose infusions (approximately30–40 μg/h/kg) of the neuropeptide resulted in initial stimulation of GnRH release that is followed by desensitization of KISS1R and a hypogonadotropic state.[66]

It should be noted that there are reports in the human literature that are difficult to reconcile with the hypothesis that the output of the pulse generator to the GnRH neurons is provided by intermittent kisspeptin signaling. Studies of normal and hypogonadotropic men have reported that continuous kisspeptin infusions (kisspeptin-10, 1.5–4.0 μg/kg/h for 12–22.5 h) were associated with an increase or restoration, respectively, in frequency of pulsatile LH secretion,[67,68] and a pulsatile pattern of low amplitude LH release has been reported in one female patient with a loss of function mutation of KISS1R.[69] Identification of LH pulses, however, is somewhat subjective, and transfection of cell lines in vitro to

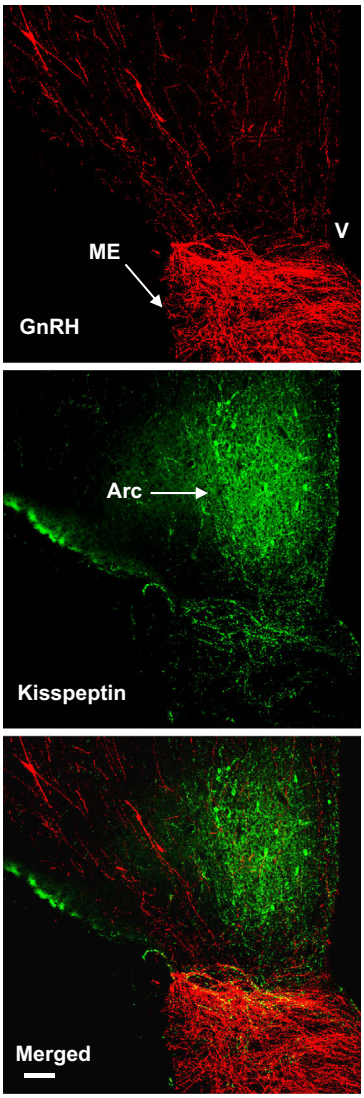

FIGURE 28.3 A hemi-coronal section through the mediobasal hypothalamus of a castrated rhesus monkey at the level of the arcuate nucleus (Arc), the posited site of the gonadotropin-releasing hormone (GnRH) pulse generator. This figure is reproduced in color in the color plate section. The section was doubled labeled by immunofluorescence for GnRH and kisspeptin. Top panel, a confocal image of the distribution of GnRH cell bodies and fibers; middle panel, also a confocal image of the same section showing the corresponding distribution of kisspeptin cell bodies and fibers. The lower panel shows the merged image. Castration results in an upregulation of kisspeptin expression that is associated with robust GnRH pulse generator activity. Note that GnRH cell bodies at this level of the hypothalamus are found lateral to the kisspeptin perikarya in Arc: both sets of neurons send projections to the median eminence (ME) where they closely intermingle. The ependymal lining of the third ventricle (V) may be seen on the right-hand side of the section. Scale bar 100 μm. *Source: Rearranged from Ref. 57, with permission, Copyright 2008, The Endocrine Society.*

examine signal transduction by mutated receptors may not fully reflect the situation in the in situ GnRH neuron. Further examination of these questions is therefore required, because if the observations in man were to be substantiated they would challenge the model for GnRH pulse generation that we have developed here.

Whether the posited intermittent kisspeptin output of the arcuate pulse generator drives pulsatile secretion of GnRH by an action on the GnRH fibers in the median eminence or by targeting GnRH cell bodies and dendrites is not clear. However, the dense field of GnRH terminals in the median eminence throughout which kisspeptin axons transverse (Figure 28.3) would appear to be an ideal site at which synchronized activity of a diffusely distributed network of GnRH perikarya could be established. The potential for such control has been demonstrated by the findings that retrochiasmatic explants of rat hypothalami containing few if any GnRH cell bodies continue to release GnRH in a pulsatile mode,[70,71] and kisspeptin stimulates GnRH release directly from GnRH terminals in MBH explants from mice.[72] Axoaxonic synapses to GnRH neurons in the median eminence are rare,[73] but volume transmission by diffusion of kisspeptin in extracellular fluid to extrasynaptic KISS1R on GnRH terminals in the median eminence is possible. Consistent with this notion is the general finding across species that IV-administered kisspeptin (kisspeptin-10) is a potent stimulator of GnRH release (see Chapter 11), in spite of the fact that this form of kisspeptin does not appear to cross the blood–brain barrier[74] and is therefore unlikely to gain access to the majority of GnRH cell bodies in the hypothalamus.

A study of sheep in 2007 was the first to demonstrate that many kisspeptin neurons in the arcuate nucleus also express neurokinin B (NKB),[75] a member of the tachykinin family of peptides.[76] In most species including the monkey and human, the majority of kisspeptin neurons in the arcuate nucleus also appear to express NKB,[77,78] although the relative number of kisspeptin and NKB immunopositive cell bodies in the arcuate nucleus has been reported to differ markedly across species.[79–81] The full significance of the coexpression of kisspeptin and NKB in arcuate neurons, however, went largely unrecognized until Topaloglu and his colleagues reported in 2009 that loss of function mutations in either this ligand or its receptor (NK3R) were associated with a phenotype similar to that reported earlier for inactivating mutations of KISS1R, i.e., hypogonadotropic hypogonadism and delayed puberty.[82] As is to be expected from the clinical observation, IV bolus injection of NKB to the juvenile monkey elicits a GnRH-dependent LH discharge.[78] This action of NKB on GnRH release is upstream from that of kisspeptin as demonstrated by the finding in the monkey that LH discharges induced by an NKB agonist (senktide) are abolished by desensitization of KISS1R, but antagonism of NK3R does not interfere with kisspeptin-induced LH release.[83] The realization that two neuropeptides, each seemingly critical for driving gonadotropin secretion, were expressed by the same arcuate neurons

further focused attention on the arcuate nucleus as the critical hypothalamic regulator of GnRH release.

A third peptide, the endogenous opioid dynorphin, is also expressed in kisspeptin/NKB neurons, and therefore the acronym KNDy is commonly used to describe these unique arcuate neurons.[84] Dynorphin signals primarily at the kappa opioid receptor,[85] and, interestingly, it has been known for more than two decades that administration of naloxone, a general opiate antagonist, results in an acceleration in LH pulse frequency during the luteal phase of the menstrual cycle in women and monkeys (see later), indicating that endorphin signaling is involved in the control of GnRH pulse frequency.[86,87] The appreciation of the importance of these specific arcuate neurons in GnRH pulse generation in the rat has been cemented by the elegant finding from Rance's laboratory that ablation of KNDy neurons following NK3R-mediated uptake of the ribosome inactivating toxin, saporin, results in a loss in hypothalamic drive to LH secretion.[88]

In the hypothalamus of men, only a very small percentage of arcuate neurons that dual label for kisspeptin and NKB have been found to also express dynorphin.[80] The functional significance of this and other species differences in the relative immunoexpression of these three arcuate peptides is difficult to ascertain because staining intensities depend on many aspects of the immunohistochemical procedures employed and, in particular, on the relative characteristics of the primary antibodies used. For these reasons, it would seem reasonable to conclude from the extant data that the role of KNDy neurons in GnRH pulse generation is likely to be fundamentally similar across mammalian species.

The neurobiological mechanisms within the arcuate nucleus that underlie the pulsatile kisspeptin output from KNDy neurons to the GnRH neuronal network remain the subject of speculation. Based on studies primarily in mice and sheep, however, it is posited that GnRH pulse generation is achieved by reciprocating stimulatory (NKB) and inhibitory (dynorphin) connections between KNDy neurons within the arcuate nucleus.[79,81,89] The evidence for this contemporary model of hypothalamic GnRH pulse generation within the arcuate nucleus has been presented in detail in Chapter 27. It should be noted, however, that this model is likely to be modified in the future. In this regard, substance P, also a tachykinin, has recently been reported to be colocalized with NKB in KNDy neurons in the human hypothalamus,[90] and studies utilizing hypothalamic slices from transgenic mice indicate that this neuropeptide, like NKB, is able to depolarize KNDy neurons.[91,92] Moreover, the stimulatory action of NKB on KNDy neurons occurs via activation of all three tachykinin receptors (NK1R, NK2R, and NK3R)[91]—a finding consistent with the in vivo observation in ovariectomized rats that pulsatile LH secretion was inhibited by central injection of a nonselective tachykinin antagonist but not by single administration of specific antagonists to the three receptor subtypes.[93] Thus, the intermittent intra-KNDy neuron stimulus that is posited to be responsible for pulsatile GnRH release in the current model of pulse generation may involve multiple and redundant tachykinin signaling pathways.

FOLLICULOGENESIS

Preantral Follicular Development

Figure 28.4 illustrates the stages of follicular development from the primordial to the preovulatory stages. Folliculogenesis begins with the transition of primordial follicles, which are formed during fetal life and may remain dormant within the ovaries for as long as 50 years, to a primary follicle. This transition is characterized by the expansion of the epithelial-like granulosa cells to a cuboidal appearance, the initiation of proliferation of the granulosa cells, and the onset of the growth of the oocyte. Up until the past few years, the mechanisms responsible for the activation of primordial follicles were a mystery. The serendipitous finding that knockout of the forkhead transcription factor, FOXO3, in mice leads to global activation of primordial follicles[94] has focused attention on the role of the phosphoinisitol 3-kinase (PI3-K) pathway as an important regulator of follicle activation. Pharmacological stimulation of the PI3-K pathway in human ovaries leads to activation of primordial follicles indicating that this pathway may also be involved in this aspect of folliculogenesis in primates.[95] A role for the PI3-K pathway specifically in preantral folliculogenesis in the human has been investigated in a recent study performed in women with premature ovarian insufficiency (POI).[96] Ovarian tissue was collected from women with POI and the PI3-K pathway was pharmacologically activated in combination with cutting the ovary into small fragments, a procedure that inactivates the Hippo signaling pathway, which in turn stimulates the growth of the ovarian fragments. Following autotransplantation of the ovarian fragments and gonadotropin therapy, mature oocytes were collected and fertilized, and embryo transfer resulted in a pregnancy and a live birth. Although very preliminary, these findings indicate that the rapidly advancing knowledge base regarding the signaling pathways involved in preantral folliculogenesis may lead to novel treatment for infertility. However, the recent observation that FOXO3 does not appear to be expressed in rhesus monkey, baboon, chimpanzee, or human oocytes questions the role of this factor in primordial follicle activation in primates.[97] Whether there are redundancies in the roles of the individual FOXO transcription factors between rodents and primate oocytes or

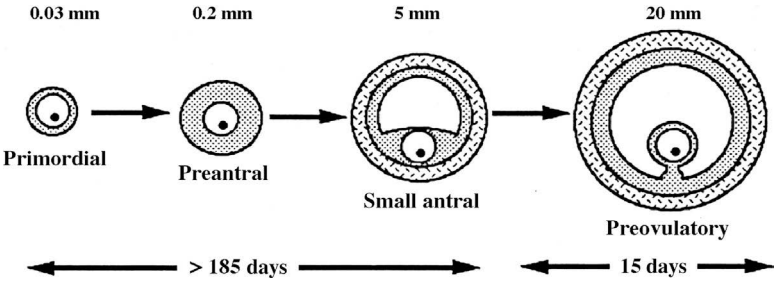

FIGURE 28.4 Pattern of human follicular development. Growth of follicles from the primordial (approximately 0.03 mm diameter) to the small antral stage (4–5 mm diameter) is characterized principally by proliferation of granulosa cells and takes greater than 120 days to complete. The maturation of a small antral follicle to a preovulatory follicle (approximately 20 mm diameter) takes approximately 15 days. See Ref. 98 for more details.

whether other downstream targets of the PI3-K pathway may control primordial follicle activation in primates remains to be established.

The role of the PI3-K pathway and other potential regulators of primordial follicle activation and preantral folliculogenesis are discussed in greater detail in Chapter 21.

After the preantral follicle acquires six to seven layers of granulosa cells, the antral cavity begins to form and the theca cell layer begins to develop. Based on measurements of mitotic indices and granulosa cell doubling times, Gougeon[98] estimated that the growth of a follicle from the primordial to the early antral stage in humans takes at least 120 days, whereas the final stages of follicular growth occur during the follicular phase of the menstrual cycle, which lasts approximately 14 days.

Histological studies have demonstrated the presence of preantral and early antral follicles in ovaries removed from hypophysectomized and prepubertal monkeys, indicating that the early stages of follicular development up to and including the formation of early antral follicles are independent of the secretion of the pituitary gonadotropins FSH and LH.[28,99,100] Similarly, morphological studies in humans and macaques identified all stages of preantral follicles throughout the entire menstrual cycle.[101,102] Use of [^{3}H]-thymidine to label dividing granulosa cells in monkeys revealed that all stages of preantral follicles undergo DNA synthesis during the luteal phase of the menstrual cycle as well as during the mid- through late follicular phases after the selection of the dominant follicle,[103] and are therefore presumably growing.

Granulosa cells from early antral follicles isolated during the human menstrual cycle do not produce substantial amounts of estradiol under basal conditions, but the secretion of estradiol can be stimulated by prolonged incubation of the cells with FSH and androgen substrate, whereas they do not respond to LH, either acutely or after prolonged incubation.[104] The responsiveness of developing monkey follicles to the gonadotropins parallels the distribution of cell surface receptors for FSH and LH because it has been demonstrated that, like that of other subprimate species, granulosa cells of preantral and early antral follicles possess cell surface receptors for FSH but not for LH.[105] Because early antral follicles are responsive to FSH and are present throughout the menstrual cycle as a result of the continuous entry of primordial follicles into the pool of developing follicles, it is generally accepted that the process of preantral follicular development serves to provide a constantly available source of maturing follicles for final development to the preovulatory stage when provided with appropriate gonadotropic support. This is best evidenced by the observations that preovulatory follicular development is rapidly stimulated in hypophysectomized monkeys and humans by the administration of exogenous FSH and LH[28–30] as well as in prepubertal monkeys by the administration of pulsatile GnRH.[106]

Antral Follicular Development

The maturation of follicles beyond the early antral stage is under obligatory gonadotropic control. During this phase of development, striking structural and functional changes occur within the follicle that result in its ability to produce steroid hormones and the ability to undergo ovulation and luteinization in response to the mid-cycle gonadotropin surge. Because preantral follicles possess only FSH receptors, it is this gonadotropin that is responsible for the initiation of preovulatory follicular development and the associated biochemical changes within the follicle that accompany this process. Microarray analysis of rat granulosa cells stimulated by FSH for 48 h revealed that over 400 mRNA transcripts are increased at least two-fold by FSH.[107] Included among the mRNAs induced by FSH are cholesterol side chain cleavage cytochrome P450 (P450scc), aromatase cytochrome P450 (P450arom), steroidogenic acute regulatory protein (StAR), and the LH receptor. The coordinated expression of P450arom results in the ability of the follicle to produce estradiol, whereas the acquisition of the LH receptors on granulosa cells confers LH responsiveness to the follicle that results in its capacity to ovulate and luteinize in response to the mid-cycle gonadotropin surge.[108]

As reviewed in detail in Chapter 20, the cAMP/protein kinase A (PKA) intracellular signaling pathway is activated by FSH in granulosa cells and appears to be sufficient to account for the dramatic increase in gene expression that accompanies granulosa cell differentiation. Thus, transduction of undifferentiated rat

granulosa cells with a lentiviral vector that expresses a constitutively active catalytic subunit of PKA indicated that a majority of genes induced by FSH were also induced to a similar extent by PKA.[107] However, a subset of genes, including those that encode for the LH receptor and P450AROM, were induced to a greater extent by FSH than by PKA, suggesting that additional signaling pathways activated by FSH may contribute to optimal gene regulation that accompanies preovulatory follicle differentiation. One likely candidate is the PI3-K/AKT signaling pathway (Chapter 20).

Control of Follicular Estradiol Biosynthesis

Gonadotropic Hormones

The classical studies of Greep et al.[21] demonstrated in hypophysectomized rats that although highly purified FSH was able to stimulate follicular growth, estradiol secretion, as measured by increases in uterine weights, was not stimulated unless LH was also administered. Administration of LH alone to hypophysectomized rats stimulated the repair of interstitial tissue but did not stimulate follicular growth or estradiol secretion. These results, published over 70 years ago, provide the framework for virtually all of our fundamental knowledge regarding the hormonal control mechanisms that govern preovulatory follicular development in primates and subprimate species.

The production of estradiol by the maturing follicle requires the interaction between the theca cells and the granulosa cells.[109] As revealed from studies conducted initially in subprimate species, theca cells under LH stimulation produce C19 androgens (testosterone and androstenedione). Because theca cells lack significant quantities of P450AROM, theca cells cannot metabolize androgens to estradiol.[110] By contrast, granulosa cells lack the 17α-hydroxylase, C17, 20 lyase activities (P450c17) that are required to metabolize C21 steroids to C19 androgens but under FSH stimulation acquire P450AROM that converts C19 androgens to estradiol.[104] Thus, Greep's original observations that both FSH and LH are required for estradiol production can be explained by the requirements for theca cell production of androgens under the influence of LH as well as granulosa cell aromatization of androgens to estradiol under the influence of FSH.

This "two-cell, two-gonadotropin" model for estradiol biosynthesis is also operative in primates. Autoradiographical studies have demonstrated that theca cells of early antral and antral follicles possess LH receptors, whereas granulosa cells contain FSH receptors.[105] Immunocytochemical analysis has demonstrated that theca cells, but not granulosa cells, possess P450c17, whereas granulosa cells from preovulatory follicles, but not theca cells, stain positive for P450AROM.[111] In vitro studies using radiolabeled steroids demonstrated minimal production of estradiol when theca cells or granulosa cells were incubated alone, whereas combining the two cell types resulted in significant production of estradiol.[112] The requirement for androgen as a substrate for estradiol production by granulosa cells was demonstrated by the findings that addition of testosterone or androstenedione to isolated human granulosa cells resulted in estradiol secretion, whereas granulosa cells incubated in the absence of androgen produced minimal amounts of estradiol.[113,114]

In humans, the FSH-mediated induction of P450AROM is the rate-limiting step in follicular estradiol secretion. Granulosa cells collected from immature follicles throughout the follicular and luteal phases produced negligible quantities of estradiol when incubated in the presence of androgen substrate unless they were exposed to FSH for 48 h.[104] In contrast, granulosa cells collected from mature preovulatory follicles were able to metabolize androgens to estradiol immediately upon removal from the follicle in the absence of added FSH.[115] In vivo, thecal androgen production does not appear to be limiting for the secretion of estradiol because androgen concentrations in the follicular fluid of early antral follicles whose granulosa cells lack P450AROM are similar to those of mature follicles whose granulosa cells possess this enzyme.[116–118]

Autocrine and Paracrine Factors

Although FSH and LH are the primary regulators of ovarian cellular function, it has become increasingly apparent that the ovarian cellular responses to the gonadotropins can be modified either in a positive or a negative manner by factors that are produced and have actions within the follicle. Those factors that have received the most attention with respect to the primate ovary include steroid hormones (androgens and estradiol), insulin-like growth factor (IGF) I, and the inhibins and activins.

STEROID HORMONES

The ability of estradiol to augment the actions of FSH on granulosa cells is well recognized, and it has been postulated that elevated estradiol concentrations present within the maturing follicle may contribute to the selection of the preovulatory follicle. This thesis is based largely on studies in rodents that demonstrated that treatment of hypophysectomized rats with estradiol or diethylstilbestrol greatly enhanced the responses of the ovary to exogenously administered FSH.[119–121] However, rigorous studies in macaques failed to demonstrate a synergistic effect of diethylstilbestrol on FSH-stimulated follicular development in vivo.[122] Messenger RNA for the estradiol receptor (ER) and immunoreactive ER protein

has been identified in baboon granulosa cells.[123] Specific antibodies that distinguish between the alpha and beta forms of the ER demonstrated that ERβ is localized to the granulosa cells of the follicle while the ERα is present in the theca cells.[124,125] Despite the presence of ERs in the follicle, it does not appear that estradiol is absolutely required for gonadotropin-dependent antral follicular development. Women with inactivating mutations of P450AROM cannot convert androgens into estradiol, and as a result, serum FSH levels are elevated due to a lack of estradiol negative feedback (see following).[126,127] Studies on the morphology of ovaries of a P450AROM-deficient girl have revealed the presence of numerous large antral follicles with intact oocytes and normal-appearing granulosa cells that regressed after the suppression of FSH secretion by the administration of low doses of estradiol.[128] These findings indicate that lack of estradiol production does not inhibit FSH-stimulated antral follicular development. However, it remains to be determined whether the large follicles present in the ovaries of P450AROM-deficient humans are functionally normal and can ovulate in response to exogenous LH or human chorionic gonadotropin (hCG).

As mentioned above, androgens are produced by theca cells under LH stimulation and are metabolized to estradiol by granulosa cells. In addition to serving as the obligatory substrate for P450AROM, androgens potentiate FSH-stimulated induction of P450AROM activity and inhibin production by primate granulosa cells in vitro.[129,130] Androgen receptor (AR) has been identified by immunocytochemistry in granulosa cells of follicles in the primate ovary, with preantral and early antral follicles exhibiting higher levels of AR than preovulatory follicles.[131] In humans, it does not appear that androgens are absolutely essential for follicular growth because successful ovarian stimulation by FSH was achieved in a woman with P450c17 deficiency, and granulosa cells isolated from this woman were able to produce estradiol when provided with an androgen substrate.[132,133]

Although androgens may not be obligatory for follicular development, studies in macaques have suggested that androgens appear to be positive regulators of this process because treatment of rhesus monkeys with testosterone or dihydrotestosterone increases the abundance of mRNA for the FSH receptor in granulosa cells and increases the growth of small follicles.[134,135] However, when the actions of androgens on gonadotropin-stimulated ovarian function were tested in vivo in rhesus monkeys, no apparent stimulation of ovarian function was observed.[136] The ability of androgens to augment FSH-stimulated follicular growth in humans has been investigated in the context of ovarian stimulation for assisted reproductive technologies, particularly in women who previously exhibited a suboptimal response to ovulation induction therapy. The results of one study did not show a beneficial effect of androgen pretreatment on FSH-stimulated follicle growth,[137] while others demonstrated that androgen pretreatment either increased the number of recruited follicles[138] or improved the apparent ovarian sensitivity to FSH, based upon the amount of exogenous FSH necessary to stimulate follicle development.[139] An inherent difficulty in evaluating potential effects of androgens on follicular development is that it is not known to what extent peripheral administration of androgens results in physiologically relevant androgen levels within the follicle. To circumvent this problem, women were pretreated with LH prior to initiating ovarian stimulation with FSH, the rationale being that LH pretreatment would stimulate endogenous androgen production by theca cells and thereby provide a more physiologically relevant intrafollicular androgen environment to the developing follicle. The results of one study[140] indicated that the number of small antral follicles and the number of normally fertilized oocytes was increased by LH pretreatment, while the results of another study[141] indicated that LH pretreatment decreased the daily amount of FSH necessary to stimulate follicle growth (improved the sensitivity to FSH) and increased estradiol concentrations but did not increase the number of maturing follicles. Collectively, the aforementioned studies suggest that androgens, although perhaps not essential for FSH-induced follicular development, may function to increase the sensitivity of follicles to FSH thereby augmenting follicular growth, as might be predicted by the findings in macaques that androgens increase FSH receptor mRNA levels in developing follicles.[134]

INSULIN-LIKE GROWTH FACTORS 1 AND 2

IGF1 synergizes with FSH in the induction of P450AROM activity in primate granulosa cells as well as with LH in the production of androgens by theca cells.[142–145] As such, IGF1 could contribute to the regulation of estradiol biosynthesis and follicular development. Although IGF1 is produced by rat granulosa cells,[146] evidence to date in primates suggests that IGF1 is not produced in human granulosa cells.[147] However, IGF1 is present in follicular fluid, presumably derived from ultrafiltration of plasma, and could thus serve as a regulator of gonadotropin action on the maturing follicle.[148]

The hypothesis that IGF1 augments the actions of FSH and/or LH on the maturing follicle was tested in a nonhuman primate animal model. Rhesus monkeys received an intravenous infusion of IGF1 for 5 days before and continuing throughout a 15-day regimen of pulsatile infusion of FSH and LH.[149] Serum estradiol and serum androstenedione concentrations were measured as an index of FSH and LH actions, and ovarian weights were measured at the end of the FSH/LH infusion. Results demonstrated that there was no augmentation of

FSH/LH-stimulated estradiol or androstenedione production by IGF1 and there was no increase in ovarian weights compared with FSH/LH treatment alone. The lack of effect of IGF1 in this in vivo setting is consistent with results from an IGF1-deficient Laron dwarf, which indicated that these subjects respond normally to ovulation induction with exogenous gonadotropins.[150]

Messenger RNA for IGF2 is present in human granulosa cells, and human granulosa cells secrete IGF2 in vitro,[151,152] suggesting that this peptide could also fulfill the role of an autocrine/paracrine agent. As reviewed by Kwintkiewicz and Giudice,[153] the biological activity of IGF2 within follicles is governed by both its production as well as its sequestration by IGF binding proteins (IGFBPs). Studies with follicular fluid isolated from healthy and atretic human follicles revealed that atretic follicles have low levels of IGF2, high levels of IGFBPs, and low levels of IGFBP protease activity. Conversely, dominant follicles have high levels of IGF2, high levels of IGFBP-4 protease activity, and low levels of IGFBPs. Collectively, these differences would result in a greater bioavailability of IGF2 in dominant follicles when compared with atretic follicles. Enhanced IGF2 signaling in the dominant follicle could amplify the biological actions of FSH and LH and therefore participate in the process of follicular development and selection. However, it should be noted that both IGF1 and IGF2 signal through the type 1 IGF receptor and, as noted above, studies in monkeys failed to reveal a significant effect of IGF1 on FSH/LH-stimulated estradiol levels and ovarian weights. Clearly, additional in vivo studies are necessary to determine if IGF1 and/or IGF2 play a major role in primate folliculogenesis.

INHIBIN/ACTIVIN

The inhibins and activins are proteins composed of two of three peptide subunits, an α subunit, a β_A subunit, and a β_B subunit. Assembly of an α subunit with a β_A subunit results in the formation of inhibin A, and assembly of an α subunit with a β_B subunit results in inhibin B. Assembly of two identical β subunits results in the formation of the homodimeric activins (activin A and activin B), whereas assembly of a β_A subunit and a β_B subunit results in activin AB.[16] The expression of inhibin and activin in the primate ovary is developmentally regulated. In cynomolgus monkeys, neither the α subunit nor the β subunits are present in preantral follicles, whereas granulosa cells in early antral follicles intensely express mRNA for the β_B subunit. As early antral follicles mature to the preovulatory stage, the expression of the β_B subunit is extinguished, whereas the expression of the α and the β_A subunits is stimulated.[154] These observations suggest that the early stages of antral follicular development may be influenced by activin, whereas the final stages of preovulatory follicle development may be influenced by inhibin. Similar findings have been reported in humans.[155] One potential role for the inhibin/activin system in the primate ovary may be the coordination of the functional activities of the theca and the granulosa cells in the production of estradiol. As mentioned above, the production of estradiol requires the synthesis of androgens by the theca cells under LH control and the metabolism of androgens to estradiol by granulosa cells in which P450AROM activity is induced by FSH. Studies with isolated human theca cells have revealed that activin antagonizes the stimulatory effects of LH on androgen production, whereas inhibin potentiates the ability of LH to stimulate androgen production.[144,156] These actions may serve to ensure that appropriate amounts of androgen are produced by the theca cells relative to the extant P450AROM activity. Before stimulation by FSH and before the induction of P450AROM, granulosa cells may produce activin, which would suppress theca androgen production and prevent excessive androgen secretion. As the follicle is stimulated by FSH, P450AROM is induced as is the ability of the follicle to produce inhibin. The increased demand for androgens by granulosa cells of the maturing follicle for subsequent aromatization to estradiol could thus be met by the actions of inhibin, which augment LH-sensitive theca androgen production. In addition to its actions on theca cell androgen production, activin also amplifies FSH-stimulated P450AROM activity in granulosa cells from immature marmoset follicles,[157] which would further enhance the ability of the developing follicle to produce estradiol. These results with primate ovarian cells showing that activin amplifies the stimulatory actions of FSH on P450AROM are similar to results obtained in rats that demonstrated that activin synergizes with FSH to stimulate DNA synthesis, as well as the induction of P450AROM and LH receptors on granulosa cells.[158,159] Collectively, these findings indicate that activin, which is expressed in FSH-dependent early antral follicles, amplifies the effects of FSH during preovulatory follicle development.

Gonadotropic Control of Preovulatory Follicular Development

Follicle-Stimulating Hormone

The essential role for FSH in preovulatory follicular development in primates was first demonstrated unequivocally by Rabin et al.[160] in their investigations of a woman with an isolated deficiency of FSH. This "experiment of nature", a 22-year-old with primary amenorrhea, had undetectable concentrations of FSH (<3 mIU/ml), elevated levels of LH (50–90 mIU/ml), estradiol levels less than 10 pg/ml, and no evidence of antral follicular development assessed by laparoscopy

and ovarian biopsy. Although initial attempts to restore ovulation in this individual with exogenous human menopausal gonadotropins were unsuccessful due to the production of anti-FSH antibodies in response to the "foreign" antigen, subsequent studies in which increasing amounts of FSH were administered to overcome the binding of the hormone to anti-FSH antibodies resulted in follicular development, estradiol production, ovulation, and successful pregnancy.[161] The low estradiol concentrations and lack of follicular development in the presence of elevated LH supports the findings of Greep et al.[21] that LH, in the absence of FSH, is unable to initiate preovulatory follicular development and estradiol secretion. The failure to observe follicular development until FSH concentrations were restored despite the presence of LH in the circulation confirmed the absolute requirement of FSH for preovulatory follicular development and estradiol secretion. Further confirmation of the absolute role for FSH in preovulatory follicular growth was revealed by the identification of additional women with isolated FSH deficiencies as well as women with inactivating mutations of the FSH receptor that are associated with the failure of ovarian follicles to advance beyond the early antral stages of maturation.[162,163]

Luteinizing Hormone

In patients with gonadotropin deficiency (low FSH and low LH), treatment with pure FSH obtained by recombinant DNA technology[164] has permitted investigations of actions of FSH in vivo in the apparent absence (or very low circulating levels) of LH. As would be predicted from Greep's observations,[21] in these hypogonadotropic patients administration of pure FSH promoted large antral follicular development but did not result in the stimulation of estradiol secretion[165] underlying the role of LH in driving thecal androgen production. In addition, inactivating mutations of the LH receptor have been identified in humans.[162,163,166] The ovarian phenotype in these individuals is exactly what would have been predicted from the extant knowledge of the effects of LH on the maturing follicle. Thus, women with inactivating mutations of the LH receptor are hypoestrogenic and anovulatory.

SELECTION OF THE PREOVULATORY FOLLICLE

The fact that numerous follicles can be stimulated to mature in humans and nonhuman primates when exogenous gonadotropins are administered eliminates the possibility that only a single follicle is available for maturation as the follicular phase commences. Although it is indisputable that the pituitary gonadotropins are indispensable for preovulatory follicular growth, why is it that a single follicle usually matures in view of the fact that FSH and LH are presumably provided to all follicles by the circulatory system? The process of follicular selection must require the operation of a highly stringent regulatory process, the function of which is to ensure that only one of the many follicles with the potential to mature to the preovulatory state actually does so. In simple terms, follicle selection must involve a mechanism by which the presence of a maturing follicle in some manner inhibits the growth of other follicles without compromising its own development.

Cessation of Preovulatory Follicular Development during the Luteal phase of the Menstrual Cycle

During the luteal phase of the menstrual cycle, preantral follicles continue to develop, but the maturation of follicles beyond the early antral stage does not occur.[101–103] Upon the regression of the corpus luteum, antral follicle growth resumes and ovulation occurs approximately 14 days later. That the hiatus in preovulatory follicular development during the luteal phase of the menstrual cycle is due to the presence of the corpus luteum has been demonstrated by the observations that removal of the corpus luteum before the expected time of luteal regression resulted in the prompt resumption of preovulatory follicular development and ovulation approximately 14 days later.[167–169] The nature of inhibitory influences imposed by the corpus luteum upon preovulatory follicular development has been the subject of debate. In monkeys, because restoration of preovulatory follicle development after luteectomy was not necessarily followed by a statistical increase in FSH and LH secretion, it was proposed that the corpus luteum directly inhibited follicular development by reducing the ability of follicles to respond to gonadotropins.[167] In humans, however, resumption of follicular development after the removal of the corpus luteum was preceded by a clear elevation of FSH concentrations.[168,169] Although the hypothesis that the corpus luteum directly inhibits follicular growth has not been disproved, the available evidence to date suggests that the corpus luteum indirectly inhibits preovulatory follicular development by way of its secretions of estradiol, progesterone, and possibly inhibin, which suppress FSH secretion (see later). Thus, as mentioned above, preantral follicular development continues throughout the luteal phase, and hence the inhibitory influences imposed by the corpus luteum must be confined to the final stages of preovulatory folliculogenesis. The observations in monkeys that preovulatory follicular development can be readily stimulated during the luteal phase of the menstrual cycle either by direct administration of exogenous gonadotropins or by interfering with the feedback inhibition of estradiol and

progesterone upon gonadotropin secretion is consistent with the hypothesis that the failure of follicles to mature beyond the early antral stages during the luteal phase is due to insufficient gonadotropin concentrations.[170,171] Finally, although systemic administration of progesterone, with its resultant feedback effects on gonadotropin secretion, after luteectomy in monkeys prolonged the interval until the next ovulation, local elevation of intraovarian progesterone concentrations by progesterone-imbedded Silastic wafers, which presumably was insufficient to restrain gonadotropin secretion, was without effect.[172]

Initiation of Preovulatory Follicular Development

Primates are unique with respect to the long duration required for the maturation of the preovulatory follicle when compared with other species, such as sheep and cows. Baird et al.[173] proposed that the lengthy follicular phase of primates is because the primate corpus luteum, unlike that of sheep and cows, produces estradiol that suppresses FSH secretion below the level necessary to advance the development of early antral follicles. Both sheep and cows display waves of follicular development during the luteal phase in which antral follicles develop but do not ovulate, presumably because the secretion of progesterone by the corpus luteum blocks the ability of estradiol that is produced by these follicles to initiate a preovulatory LH surge[173–175] (and Chapter 27). In these species, upon the regression of the corpus luteum and the removal of the feedback inhibition of progesterone on LH secretion, follicles rapidly produce estradiol, which triggers an LH surge and ovulation. By contrast, because of the suppression of FSH secretion during the luteal phase of the primate menstrual cycle, follicular development does not progress beyond the very early antral stages, hence more time is required for follicles to undergo FSH-dependent differentiation. It should be noted however that the notion that primates do not exhibit waves of follicle growth has recently been challenged by serially monitoring human ovaries using high resolution transvaginal ultrasonography throughout the menstrual cycle.[176] These studies revealed the existence of two or three waves of antral follicle growth as reflected by the periodic presence of a follicle (or follicles) with diameters equal to or greater than 5 mm (Figure 28.4). In women with two follicle waves, one wave occurred during the early follicular phase and produced the follicle destined to ovulate, and the second wave occurred during the luteal phase of the cycle, shortly after the mid-cycle gonadotropin surge. Women with three follicle waves exhibited, in addition to the wave of follicle growth during the luteal phase, two waves of follicle growth during the early follicular phase with the second wave producing the follicle destined to ovulate. The overall significance of follicle waves in humans remains uncertain in view of the observations that removal of the corpus luteum in both monkeys and humans is followed by ovulation approximately 14–17 days later,[168,169] while in sheep, ovulation can occur within 3 days of induced luteal regression.[177,178]

Upon the demise of the corpus luteum and the clear elevation in plasma FSH and LH concentrations (Figure 28.1), the maturation of the follicle that is destined to ovulate is initiated. Although the perimenstrual rise in serum FSH concentrations was recognized by Ross et al.[179] in their original descriptions of the hormonal profiles of the human menstrual cycle, the extent to which such a slight (30–50%) rise in FSH concentrations during the early follicular phase participated in follicular development was not fully appreciated until Brown[180] demonstrated that changes in FSH concentrations on the order of 10–30% are sufficient to initiate follicular development in anovulatory women.

During the follicular phase of the menstrual cycle, there are dynamic changes in the patterns of the plasma concentrations of FSH, estradiol, and inhibins A and B (Figure 28.1). The early to mid-follicular phase, before the emergence of a stimulated follicle, is characterized by elevated serum FSH concentrations and inhibin B concentrations. Serum estradiol and inhibin A concentrations are low. Approximately 7 days before the mid-cycle gonadotropin surge, serum concentrations of estradiol and inhibin A begin to increase, while inhibin B and FSH concentrations decline. The rise in serum estradiol and inhibin A concentrations during the mid through late follicular phase is likely due to the emergence of a maturing preovulatory follicle and its associated expression of P450AROM and the β_A subunit of inhibin.[110,154] Serum levels of inhibin B fall slightly, possibly due to the decline in the expression of the β_B subunit that accompanies preovulatory follicle development as well as atresia of small antral follicles.[154] Associated with this gradual increase in estradiol and inhibin A concentrations is a progressive fall in FSH concentrations due to the feedback actions of estradiol and/or inhibin A on gonadotropin secretion (see later). This classical negative feedback relationship between developing follicle and the secretion of FSH by the pituitary gland is the essential component in the process of follicular selection. Because of the steady exit of follicles from the primordial pool, there is always a maturational distinct population of early antral follicles within the ovaries ready for development to the preovulatory stage under the influence of FSH. When a growing follicle acquires sufficient P450AROM activity and/or inhibin biosynthetic capacity as a result of FSH stimulation, FSH levels are suppressed to below those necessary to promote the further development of less mature follicles, which then undergo atresia.

How Does the Maturing Follicle Inhibit the Development of Other Follicles?

As noted before, the classical negative feedback effects of estradiol and/or inhibin provide a mechanistic explanation of how the presence of a maturing follicle could curtail the maturation of other follicles by suppressing FSH secretion. Because both estradiol and inhibin A plus B concentrations are elevated during the follicular phase (Figure 28.1), it has been difficult to determine the relative importance of estradiol and the inhibins in the negative feedback regulation of FSH secretion. The ensuing sections summarize the evidence in favor of either estradiol or inhibin as being the principal ovarian feedback regulator of FSH secretion.

Estradiol

The hypothesis that the maturing follicle inhibits the development of less mature follicles by suppressing FSH secretion by way of its production of estradiol has been tested directly in humans and nonhuman primates by manipulating the pattern of estradiol concentrations during the follicular phase of the menstrual cycle. In rhesus monkeys, subcutaneous insertion of estradiol-containing capsules on days 3–6 of the follicular phase of the menstrual cycle prematurely elevated estradiol concentrations by 50–80 pg/ml and resulted in a slight but significant fall in the plasma concentration of FSH and an interruption of spontaneous follicular development. When the estradiol capsules were removed, FSH concentrations once again became elevated, whereupon follicular development began anew and ovulation occurred 14 days later.[181] Analogous studies in humans demonstrated that oral administration of ethinyl estradiol (the major component of oral contraceptives) during the early follicular phase of the menstrual cycle resulted in a fall in FSH concentrations and a lengthening of the follicular phase, an effect that was negated either by concurrent administration of an anti-estrogen[182] or by the administration of exogenous FSH.[183]

This feedback model for follicle selection would also predict that negating the gonadotropin-suppressing effects of estradiol during the mid through late follicular phases of the menstrual cycle should prevent the fall in FSH concentrations and result in the maturation of more than one preovulatory follicle. Indeed, passive immunization of rhesus monkeys with anti-estradiol antibodies during the mid through late follicular phases of the menstrual cycle prevented the fall in FSH concentrations and caused the maturation of more than one preovulatory follicle.[184] In humans, it is well known that antagonizing the biological actions of estradiol with the anti-estrogen clomiphene or inhibition of P450AROM results in an augmentation of FSH secretion and maturation of more than one preovulatory follicle.[185,186]

Inhibin

Although the aforementioned studies are consistent with the hypothesis that feedback inhibition of FSH secretion by estradiol is a major mechanism by which the maturing follicle suppresses the development of less mature follicle, as can be seen in Figure 28.1, concentrations of inhibin A and B, parallel those of estradiol during the follicular phase. Inhibin B is produced by small antral follicles and has an in vivo biopotency in ovariectomized rats that is approximately 1.45-fold greater than inhibin A, which is produced by the dominant follicle and the corpus luteum.[154,187] Because both inhibins are present in the circulation during the follicular phase, it is possible that inhibin A and/or inhibin B may also be an important regulator of FSH secretion and hence follicle selection. There are two lines of evidence that support a possible role for inhibin in the feedback regulation of FSH secretion during the follicular phase. First, a number of studies have shown that administration of porcine follicular fluid (that was treated with charcoal to remove steroid hormones) during the follicular phase of the menstrual cycle suppresses FSH secretion and interferes with the timely development of a preovulatory follicle.[188,189] However, the limitation of these studies is that it is not known whether resultant inhibin levels achieved by this treatment were within the physiological range. In addition, follicular fluid would also contain follistatin, which could interfere with activin-stimulated FSH secretion by the pituitary.[190] Administration of inhibin A during the follicular phase of the menstrual cycle was shown to suppress FSH secretion in monkeys.[191] However, because the resultant plasma inhibin level was 10 ng/ml, about 100 times greater than the combined concentrations of inhibin A and inhibin B seen during the follicular phase (Figure 28.1), it is likely that these observations reflect a pharmacological action of inhibin. It should also be noted that while inhibin B concentrations fall during the mid to late follicular phase, inhibin A concentrations rise during that interval such that the total inhibin concentrations (inhibin A + inhibin B) do not change dramatically during the mid through late follicular phase when FSH levels are declining (Figure 28.1).

A second line of evidence in support of inhibin as an important regulator of FSH secretion is the observation that FSH levels are elevated during the follicular phases of older women.[192,193] That these women have normal levels of LH and estradiol but reduced levels of inhibin B is suggestive that diminished inhibin B, reflecting the reduction in follicle number that accompanies ageing (Chapter 37), may be causal to the elevated FSH levels observed. However, in older women there is a reduction in the sensitivity to estrogen-negative feedback on FSH secretion, which could also play a role in the elevated FSH levels in these subjects.[194]

In the passive immunization studies with anti-estradiol antibodies described previously,[184] neutralization of circulating estradiol in monkey resulted in an elevation of FSH and maturation of more than one preovulatory follicle, presumably in the presence of elevated inhibin

concentrations that would have resulted from the increased number of maturing follicles.[195] In humans, administration of the anti-estrogen clomiphene resulted in an increase in both estradiol and inhibin B concentrations, but FSH levels were not suppressed.[196] These observations therefore suggest that when the negative feedback effect of estradiol is interrupted, inhibin alone is not able to suppress FSH secretion.

As mentioned above, humans with P450AROM deficiency have elevated levels of FSH in the presence of multicystic ovaries with macroscopically normal large antral follicles, which are the primary source of circulating inhibin A.[127,128] Yet despite the presumption of increased circulating inhibin A concentrations, FSH levels in these subjects were not suppressed. Moreover, when these subjects were treated with exogenous estradiol, serum FSH levels declined and the multicystic ovaries resolved.[128] In addition to knowledge gained from women with P450AROM deficiency, the recent identification of a woman with an inactivating mutation of ERα,[197] which has been shown in genetically modified mice to regulate estrogen-dependent negative feedback of gonadotropin secretion[198] has provided important additional insight into the relative roles of estradiol and inhibin in the control of FSH secretion in humans. This patient exhibited markedly elevated levels of estradiol and inhibin A and pelvic ultrasonographic analysis revealed the presence of enlarged multicystic ovaries, consistent with excessive stimulation of follicular development. Not surprisingly, this patient also had elevated levels of FSH, the presumptive cause of the excessive folliculogenesis, despite elevated levels of estradiol and inhibin A. Thus, in the absence of estrogen-mediated negative feedback, elevated levels of inhibin A were unable to adequately suppress FSH secretion to levels seen during the mid through late follicular phase of the menstrual cycle and therefore limit the number of preovulatory follicles that develop. These compelling observations from this subject indicate that estradiol, rather than inhibin A, is the principal ovarian hormone that is responsible for feedback inhibition of FSH secretion and the resultant control of mono-ovulatory follicular development in humans. However, it is also important to note that serum levels of FSH in this subject were only moderately elevated in comparison to those seen in postmenopausal women that lack both ovarian estradiol and inhibin production.[199] The novel phenotype of this subject could therefore be interpreted to indicate that inhibin may act to set the upper level of FSH secretion while estradiol regulates FSH production to achieve the additional control of serum concentrations of FSH needed to ensure that levels fluctuate within the window necessary for mono-follicular development. It is also possible that other ERs may be involved in the negative feedback control of FSH secretion (see following), which could explain why FSH levels do not rise into the postmenopausal range in this woman with ERα deficiency.

Although we conclude that the extant evidence favors a more important role for estradiol than for inhibin in regulating FSH secretion during the follicular phase of the menstrual cycle, a conclusion that was also reached by mathematical modeling (Chapter 29), in the context of the physiology of the menstrual cycle the important take-home message is that the maturing follicle inhibits the maturation of other follicles by depriving them of adequate FSH support.

How Does the Maturing Follicle Survive Its Self-imposed Suppression of FSH Secretion?

Given that FSH is absolutely required for preovulatory follicle growth and that the maturing follicle inhibits the development of other follicles by suppressing FSH secretion, how is it that the maturing follicle continues to develop in the presence of FSH concentrations that are unable to maintain the development of less mature follicles? The only explanation for this paradox is that as the follicle matures, it becomes less dependent on FSH such that the concentration of FSH necessary to initiate preovulatory follicular development is greater than the concentration of FSH necessary to maintain preovulatory follicular growth. This hypothesis was tested directly by infusion of highly purified human FSH and human LH into cynomolgus monkeys whose endogenous gonadotropin secretion was blocked by a GnRH antagonist.[200] As shown in Figure 28.5, when plasma FSH levels were maintained at approximately 10 mIU/ml, there was no evidence of estradiol secretion. Elevation of plasma FSH concentrations to approximately 20 mIU/ml resulted in the initiation of preovulatory follicular development as reflected by increasing concentrations of estradiol. Once preovulatory follicular growth was stimulated by FSH, reduction of plasma FSH concentrations to 8–10 mIU/ml over a five-day period was associated with a continued rise in estradiol production, and histological examination of the ovary revealed the presence of mature preovulatory follicles. That estradiol secretion continued to rise despite the progressive fall in FSH concentrations demonstrates that the maturing follicle, as a consequence of FSH simulation, exhibits a diminished requirement for FSH such that it continues to mature in the presence of FSH concentrations that are unable to initiate the development of less mature follicles. Similar findings have been observed in humans.[201,202] These data support the hypothesis developed by Brown[180] of a threshold concentration of FSH that must be achieved to initiate preovulatory follicle development. Not surprisingly, this experimentally determined threshold concentration of FSH is very close to the actual concentration of FSH measured during the early follicular phase of the human menstrual cycle when the initiation of preovulatory follicular development occurs.[179] Once this threshold concentration of FSH is reached, follicles readily enter the final stages of preovulatory development.

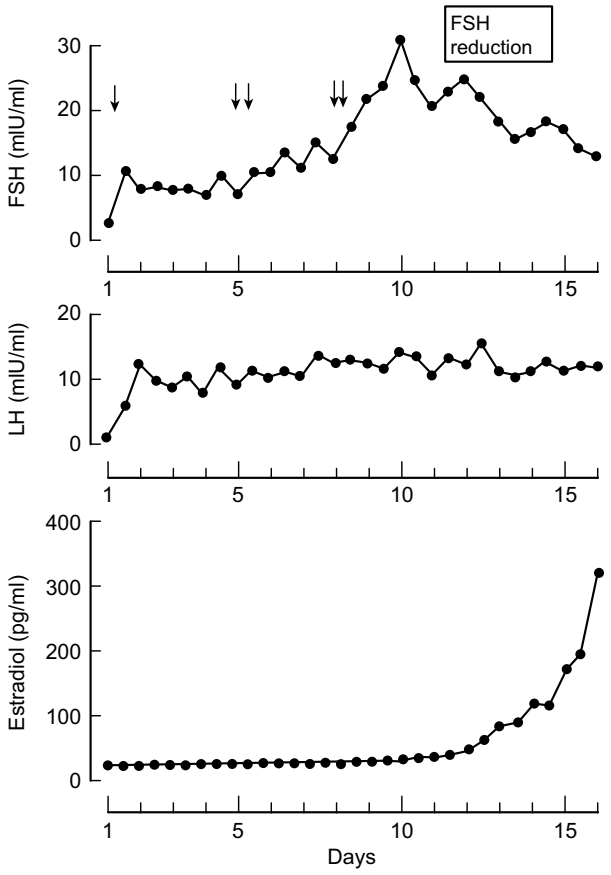

FIGURE 28.5 Ovarian responses in a cynomolgus monkey that received hourly pulses of purified human follicle-stimulating hormone (FSH) and human luteinizing hormone (LH). The concentration of FSH in the infusate was increased every 4–5 days until estrogen secretion became evident; thereafter, the concentration of FSH in the infusate was reduced by 12.5% per day for 5 days. Estrogen secretion, reflecting the presence of a maturing follicle, was stimulated when plasma concentrations reached ~20 mIU/ml. When plasma FSH concentrations were reduced to levels that were unable to initiate estrogen secretion (10–15 mIU/ml), the production of estrogen by the stimulated follicle continued to increase in an exponential manner. *Source: Reproduced from Ref. 200, with permission, Copyright 1986, The Endocrine Society.*

The number of follicles that develop to the preovulatory stage is dependent on the duration that FSH concentrations are maintained above threshold.[200,203] During spontaneous menstrual cycles, the operation of the negative feedback loop between estradiol and FSH secretion provides moment-to-moment communication between the ovary and the hypothalamic-pituitary axis that results in the suppression of FSH secretion and the cessation of secondary follicular development upon the emergence of a maturing follicle.

Although a single follicle is usually selected during the human menstrual cycle, dizygotic twinning (fertilization and implantation of two oocytes) does occur in about 1% of human pregnancies. Although it has been proposed recently that mutations in the FSH receptor that lead to increased sensitivity of the receptor to FSH could be one factor responsible for dizygotic twinning,[204] this hypothesis has been disputed because these amino acid substitutions do not confer enhanced FSH responsiveness as assessed by hormone binding and cAMP production in transfected COS7 cells.[205] There are, however, two interesting "experiments of nature" that point to increased FSH levels being associated with dizygotic twinning. The first example is that the rate of dizygotic twinning increases in women with maternal age, despite the paradoxical fact that the number of ovarian follicles declines significantly with age (Chapter 37). Beemsterboer et al.[206] used ultrasound examinations to assess the incidence of multiple periovulatory follicles (>1 follicle with a diameter of >14 mm) in women and found that basal levels of FSH obtained on the third day of the menstrual cycle were greater in 30–41 year-old women that exhibited multifollicular growth compared with those with monofollicular growth. They proposed that these elevated FSH levels may have resulted in overshooting the threshold, which could result in selection of more than one ovulatory follicle, perhaps by lengthening the time that FSH levels were maintained above threshold values.[200,203] A second example is that of Yoruba women in western Nigeria who have an incidence of dizygotic twinning that is nearly five times the average rate of other women. Serum FSH levels in these women are higher than those of a control group.[207] It has been speculated that increased consumption of local white yams that may contain estrogen-like substances could act in a similar fashion to ovulation-inducing agents (such as clomiphene) and elevate FSH levels and thereby stimulate multifollicular development.

Mechanisms by Which the Maturing Follicle Becomes Less Dependent on FSH

The final solution to the question of follicle selection requires the elucidation of the mechanism(s) by which the maturing follicle escapes its requirement for elevated FSH concentrations. During preovulatory follicular development, there are changes in the cellular functions of the FSH-stimulated follicle that may contribute the selective maturation of a preovulatory follicle. These development-dependent changes may occur at different levels, and the advantages that each may provide to the follicle are not mutually exclusive but rather could likely provide a variety of "fail safe" mechanisms that ensure the maturing follicle is spared from the reduction in FSH concentrations.

LH and FSH Receptors

Increases in the density of cell surface receptors for FSH and LH enhance the sensitivity of target cells to the hormone, as predicted by the law of mass action.

In rats, the density of FSH receptors on granulosa cells increases during preovulatory follicular development.[208] Although comparable studies have not been reported in women or monkeys because of the difficulty in obtaining sufficient amounts of granulosa cells from unstimulated follicles, analysis of the dose–response relationship between FSH concentrations and estradiol production by marmoset granulosa cells revealed that cells collected from preovulatory follicles were nearly four times more sensitive to FSH when compared with granulosa cells harvested from less mature follicles.[209] A four-fold increase in responsiveness to FSH would more than compensate for a 50% decline in plasma FSH concentrations.

In addition to changes in the number of FSH receptors on granulosa cells, a hallmark action of FSH is to induce cell surface receptors for LH.[210] The presence of LH receptors on granulosa cells of developing follicles could also serve to protect the follicle from the fall in plasma FSH concentrations by providing the follicle with the ability to respond to LH. Because preovulatory follicles are responsive to both FSH and LH with respect to increases in cAMP production, whereas early antral follicles are responsive to only FSH[104,115] (and Chapter 20), a reduction in FSH concentrations would reduce the only source of gonadotropic support to early antral follicles. By contrast, the FSH-mediated induction of LH receptors on granulosa cells could protect the mature follicle from reduced FSH concentrations by providing it with the ability to respond to both FSH and LH.

The hypothesis that the selected follicle has reduced dependence on FSH because of its acquisition of LH responsiveness was first tested in humans.[211] In this study, women whose spontaneous gonadotropin secretion was suppressed by a GnRH agonist received recombinant human FSH until at least one follicle attained a size of 14 mm. Thereafter, subjects were then randomized into three groups for a two-day period during which either FSH treatment was continued or FSH treatment was discontinued and replaced with either recombinant LH or saline. Results demonstrated that serum estradiol levels continued to rise in the women that received either FSH or LH, whereas in those receiving saline estradiol levels declined over the two-day treatment interval indicating that LH can replace FSH in the final stages of preovulatory follicle development. Subsequent studies in humans demonstrated that following FSH treatment, LH alone can maintain the maturation of a preovulatory follicle and, moreover, stimulate the formation of a single preovulatory follicle more effectively than either FSH alone or FSH given in combination with LH.[212] Studies in sheep, horses, and cows, as reviewed in Refs 213 and 214, have also indicated that a shift in responsiveness from FSH to LH likely plays a role in continued development of the dominant follicle in the presence of declining FSH concentrations. Thus, the FSH-mediated induction of LH receptors on granulosa cells may be a common mechanism that many mammalian species employ to select the preovulatory follicle.

Angiogenesis

In addition to increases in gonadotropin responsiveness, preferential delivery of gonadotropins to the maturing follicle could protect it from a fall in FSH concentrations. In rhesus monkeys, the density of the capillary network that supplies the preovulatory follicle is at least three times greater than that supplying other less mature follicles, and this increased density of capillaries results in a greater delivery of radioiodinated gonadotropin to the selected follicle.[105] The finding that extracts of follicle cells or conditioned tissue culture media from granulosa cells were able to stimulate the proliferation of endothelial cells in vitro suggested that the maturing follicle may produce diffusible angiogenic factors that cause selective vascularization of the theca layer.[215] A family of heparin-binding endothelial growth factors has been identified. The first members of the family, vascular endothelial growth factor (VEGF) and vascular permeability factor, were isolated, respectively, from bovine pituitary cell–conditioned medium and a guinea pig tumor cell line.[216,217] Human VEGF is a glycoprotein of 165 amino acids, whereas the structure of human vascular permeability factor is nearly identical to that of VEGF except it contains an additional internal amino acid sequence.[217] Additional forms of VEGF have now been identified and appear to be derived from alternate exon splicing.[218] VEGFs are secreted proteins that specifically stimulate endothelial cell proliferation, and receptors for VEGF are ubiquitous on capillary endothelial cells.[219] In situ hybridization studies on ovaries from cynomolgus monkeys have demonstrated that mRNA for VEGF was expressed strongly by the maturing follicle but not by other antral follicles, indicating this protein could be responsible for the selective vascularization that accompanies preovulatory follicular development.[220] Studies by Zimmermann et al.[221] tested the hypothesis that VEGF plays a role in follicular selection by treating rhesus monkeys with an antibody directed against the VEGF receptor and observed that administration of anti-VEGF receptor antibody, but not a control antibody, inhibited preovulatory follicular development despite the presence of elevated FSH concentrations. Similar findings in marmosets indicated that VEGF plays an essential role in preovulatory folliculogenesis.[222]

Neuroendocrine Control of FSH and LH Secretion by the Maturing Follicle

As described above, the suppression of FSH secretion during the second half of the follicular phase of the cycle, most likely mediated by the ovarian production

of estradiol, underlies the mechanism whereby the presence of the maturing follicle inhibits the development of others but does not succumb to its own inhibitory influences. In women, this decline in FSH secretion is monotropic, i.e., it is not accompanied by a reduction in circulating LH levels, whereas in the female monkey there is parallel suppression in the secretion of the two gonadotropins during the late follicular phase.[47,50] Here, we discuss the neuroendocrine bases of the negative feedback action of ovarian estradiol responsible for suppression of FSH secretion during the late follicular phase of both the human and monkey menstrual cycle. Four major aspects will be considered. First, the essential features of the negative feedback loop whereby the primate ovary restrains gonadotropin secretion throughout the greater part of the menstrual cycle will be briefly stated. Second, the relative importance of pituitary versus hypothalamic sites of feedback action by estradiol on gonadotropin secretion will be discussed. Third, the molecular and cellular mechanisms underlying the negative feedback action of estradiol will be examined. Fourth, the cell biology underlying the differential secretion of FSH and LH from the human gonadotrope will be briefly and speculatively examined.

The Classic Negative Feedback Loop between Gonadotropin Secretion and Ovarian Hormones

As described before the ovarian cycle is directed by the gonadotropins, the secretion of which is in turn governed by classic negative and positive feedback loops between the ovary and pituitary (see Chapter 33). During the menstrual cycle, the negative feedback loop restrains the secretion of both FSH and LH throughout most of the follicular and luteal phases. Thus, when this loop is opened either by ovariectomy or following menopause, circulating levels of the gonadotropins increase progressively to plateau at values an order of magnitude higher than those observed during the early follicular phase of the cycle. Reclosing the negative feedback loop in the ovariectomized monkey by replacement with estradiol that produces follicular phase levels of the circulating steroid restores gonadotropin concentrations to the low values typically observed in the early follicular and luteal phases (for full details, see Chapter 45 in the third edition).

Hypothalamic and Pituitary Sites of Negative Feedback Action of Estradiol

Theoretically, the suppression of gonadotropin secretion by estradiol may be achieved by an action of the steroid either directly at the level of the pituitary to inhibit the response of the gonadotropes to GnRH stimulation or indirectly at the level of the hypothalamus to decrease the secretion of GnRH, or by a combination of the two. In women with hypogonadotropism due to a deficiency in GnRH and in monkeys bearing hypothalamic lesions that abolish gonadotropin secretion by preventing GnRH release, menstrual cyclicity may be induced by administration of a constant intermittent infusion of GnRH[223–225] (Figure 28.6). Since the GnRH input to the pituitary gonadotropes is invariant in these experimental models, known as "hypophysiotropic clamps", the negative feedback action of estradiol responsible for the approximately normal levels of gonadotropin in the follicular phase must be exerted directly at the pituitary. Similarly, in hypogonadotropic women, estrogen administration that resulted in follicular phase levels of circulating estradiol suppressed FSH and LH in response to pulsatile GnRH stimulation for 5 days.[226] Again, in these experiments, the negative feedback action of estradiol must be exerted at the pituitary because the GnRH drive to the gland was exogenous and invariant. In a complex human model employing postmenopausal women in whom the pituitary was acutely emancipated from hypothalamic input by administration of a GnRH

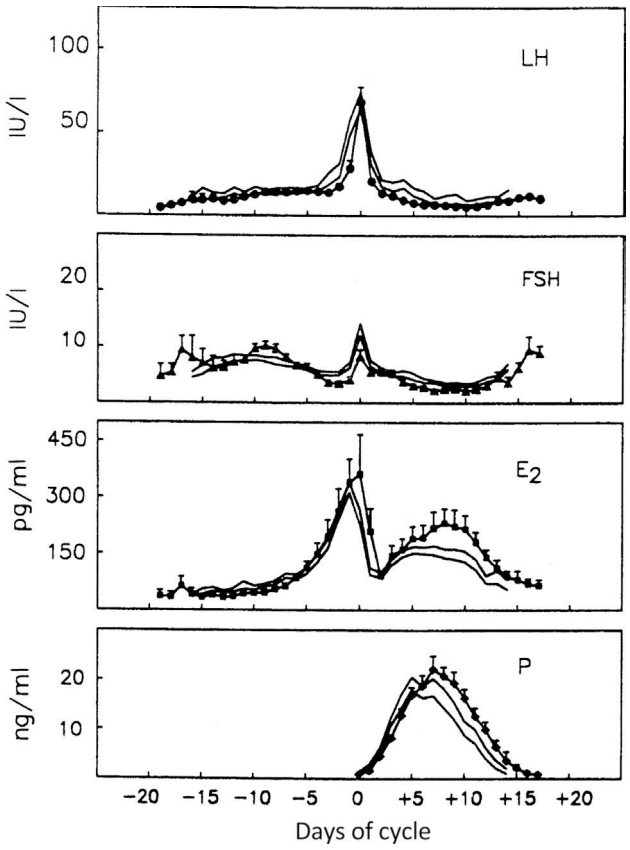

FIGURE 28.6 Induction of ovulatory menstrual cycles in women with primary hypothalamic amenorrhea with an unvarying pulsatile gonadotropin-releasing hormone (GnRH) treatment (5.0 μg GnRH pulse IV every 60 min). Note the close similarities in hormonal profiles of the women with amenorrhea and treated with GnRH and normal controls depicted by the area between the broken lines. Day 0, day of the preovulatory luteinizing hormone (LH) surge. *Source: Reproduced from Ref. 225, with permission, Copyright 1991, The Endocrine Society.*

antagonist (at a dose that did not block LH responses to large doses of exogenous GnRH), Hall and her colleagues demonstrated that the response of FSH and LH to exogenous GnRH was reduced by chronic exposure to follicular phase levels of estradiol indicating negative feedback directly at the pituitary.[194]

While the foregoing studies provide strong support for negative feedback actions of estradiol at the pituitary, there is also evidence that this steroid can inhibit gonadotropin secretion indirectly by suppressing hypothalamic GnRH release. Microinjections of estradiol into the hypothalamus or third cerebroventricle suppressed LH secretion in ovariectomized rhesus monkeys.[227,228] Peripheral administration of the steroid to ovariectomized or estrogen-replaced ovariectomized monkeys led to a rapid suppression of GnRH release in the region of the pituitary stalk and median eminence,[229] and to a deceleration or interruption in the volleys of MUA in the MBH[230,231]: episodes of hypothalamic electrical activity that are tightly coupled with pulsatile LH release (Figure 28.2) and thereby provide a robust indicator of GnRH pulse generator activity.[54] The physiological significance of the observed estradiol-induced changes in GnRH pulse generator activity in the ovariectomized monkey, however, are unclear. This is because GnRH pulse generator frequency, as reflected by MUA (monkey, Figure 28.7) and episodic LH release (monkey and human) during the last half of the follicular phase of the cycle before initiation of the LH surge is held constant at approximately 1 pulse/h, despite the progressive rise in circulating estradiol at this time.[53,232,233] It therefore seems reasonable to propose that any indirect negative feedback action of estradiol on gonadotropin secretion during the late follicular phase is mediated by a hypothalamic mechanism that results in amplitude modulation of pulsatile GnRH release. This view is supported by the finding that in ovariectomized monkeys estradiol administration leads to a reduction in GnRH pulse amplitude in the absence of a change in pulse frequency[229] and by studies of the ewe (see Chapter 27).

The relative importance of hypothalamic versus pituitary sites of negative feedback action of estradiol that underlie the restraint of gonadotropin secretion during the menstrual cycle, including the suppression of FSH secretion in the latter half of the follicular phase, is unclear. What is clear, however, is that as may be anticipated for a control system governing reproduction, a high degree of redundancy is likely to exist as indicated by the finding that gonadotropin secretion throughout the follicular phase of the menstrual cycle may be controlled by solely ovarian negative feedback directly to the pituitary, as compellingly demonstrated in hypophysiotropically clamped paradigms employing both women and female monkeys as experimental subjects[223–225] (Figure 28.6).

Cellular and Molecular Mechanisms Underlying the Negative Feedback Action of Estradiol

The cellular and molecular mechanisms underlying the direct and indirect negative feedback actions of estradiol at the level of the pituitary and brain, respectively, have not been extensively studied in primates, but as in other species are presumably mediated by activation of ER signaling pathways in either the gonadotrope or in neuronal circuits regulating the secretion of GnRH. As noted above, there are two forms of nuclear ER (ERα and ERβ), (also see Chapters 9 and 25). Targeted independent deletion of these proteins in mice has led to the view that ERα mediates the negative feedback action of estradiol on gonadotropin secretion (see Chapter 26). Recent studies of a woman with a loss

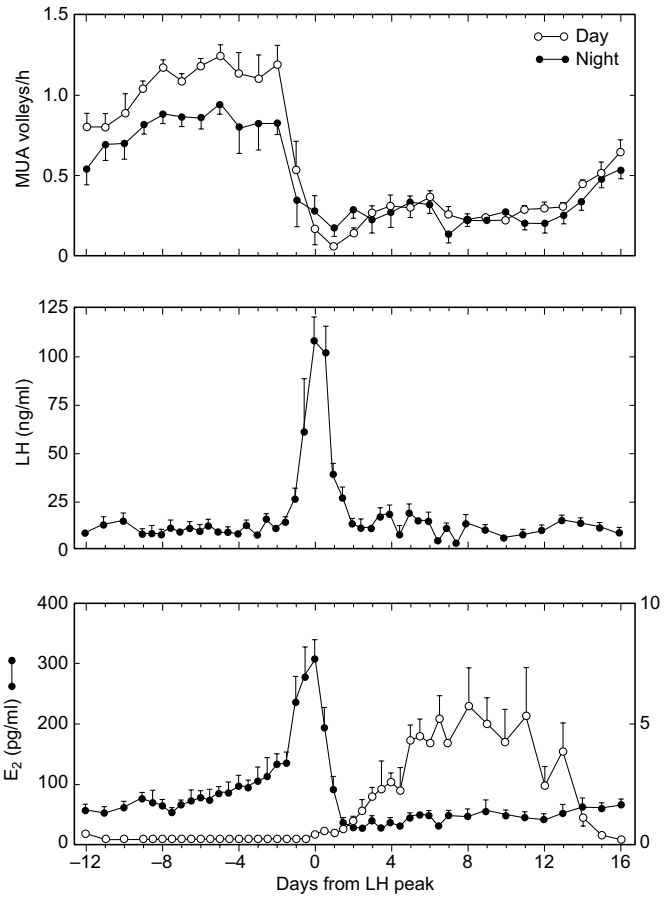

FIGURE 28.7 Frequency of gonadotropin-releasing hormone (GnRH) pulse generator activity (MUA volleys per hour) monitored by radiotelemetry during the menstrual cycle of unrestrained rhesus monkeys, with accompanying time courses of luteinizing hormone (LH), estradiol, and progesterone. Data are normalized to the day of the mid-cycle LH peak (day 0). Each point represents the mean ± standard error of the mean ($N = 5–12$ observations). Note the slower frequency of the GnRH pulse generator at night during the follicular phase, the abrupt reduction in pulse generator activity with the onset of the LH surge, and the slower frequency of the pulse generator during the luteal phase in comparison with its activity during the follicular phase. (MUA, multiunit electrical activity.) *Source: Reproduced with permission from Ref. 53, Copyright 1991, The Endocrine Society.*

of function mutation in ERα (see above) also suggest that this receptor mediates, at least in part, the negative feedback action of estradiol in the human female. Interestingly, studies of transgenic mice indicate that ERα utilizes both the classical genomic and the more rapid membrane initiated pathway (nonclassical) to modulate function of target cells mediating the negative feedback action of estradiol on gonadotropin secretion.[234]

In the context of a direct pituitary site of estradiol negative feedback, the finding in the rat that the predominant ER expressed in the anterior pituitary is ERα[235] is consistent with our understanding of the molecular basis of this action of estradiol. The relative importance of classical genomic versus nonclassical membrane-initiated ER signaling pathways in the pituitary is unclear. In this regard, a second putative membrane ER, GPR30, has been reported in anterior pituitary of rat and cow,[236,237] and further study of membrane-initiated ER signaling in the context of negative feedback action of estradiol is merited. This is because of the rapidity of this action of estradiol, which may lead to an immediate block of the hourly episodes of LH secretion in ovariectomized monkeys following the peripheral administration of the steroid.[238,239]

In the case of hypothalamic sites of estradiol negative feedback it is now generally recognized that ERα is not expressed by GnRH neurons (see Chapter 11), and this has led to the proposal that the action of the steroid in this regard must be exerted on neural and or glial systems, which are afferent to the GnRH neuronal network. While there are several such estrogen-responsive systems that can modulate GnRH secretion (see Chapters 26 and 27), attention is currently focused on the kisspeptin neurons in the arcuate nucleus. Data are at hand to support the view that these neurons represent a major hypothalamic site for the negative feedback action of estradiol on gonadotropin secretion in primates, as in other species (see Chapters 26, 27, and 33). In the hypothalamus of postmenopausal women, Rance and her colleagues in 2007 demonstrated using in situ hybridization that *KISS1*-expressing neurons were hypertrophied,[58] a neuronal phenotype previously shown by this group to express *ERa* (see Ref. 240). Moreover, the use of immunohistochemistry and in situ hybridization has shown that ovariectomy in the rhesus macaque results in an increase in kisspeptin mRNA and peptide content in neurons in the arcuate nucleus, while replacement with estradiol leads to a reduction in *KISS1* expression.[58,59] The estrogen-induced suppression of *KISS1* expression in the MBH of ovariectomized monkeys has been confirmed by RTPCR.[241] The foregoing changes in kisspeptin mRNA and peptide content in neurons of the arcuate nucleus appear to directly reflect their neurosecretory activity as ovariectomy in the pubertal monkey results in an increase in the release of kisspeptin into the region of the median eminence.[62] Studies of transgenic mice have implicated an important role of nonclassical ERα signaling in arcuate kisspeptin neurons in mediating the negative feedback action of the estradiol.[242]

The estrogen-responsive kisspeptin neurons in the arcuate nucleus are likely to be KNDy neurons, which are considered to be a fundamental component of the GnRH pulse generator (see above). In the hypothalamus of the human female, the majority of arcuate kisspeptin neurons also coexpress NKB, and NKB neurons in the arcuate nucleus also exhibit hypertrophy in postmenopausal women.[243] Similarly, the hypertrophic arcuate neurons in postmenopausal women also express the mRNA encoding for prodynorphin.[244] Taking the foregoing considerations together, it seems reasonable to propose that amplitude modulation of the GnRH pulse generator is the major mechanism underlying the negative feedback action of estradiol at the hypothalamic level: the estrogen-dependent change in pulse generator activity being relayed to the GnRH neuronal network by variation in the intermittent delivery of kisspeptin to the median eminence.

Although membrane actions of estradiol on primate GnRH neurons have been described,[245] to date these have been excitatory and are therefore unlikely to play a role in mediating negative feedback action of this steroid at the hypothalamic level.

Differential Negative Feedback of Estradiol on FSH and LH in Late Follicular Phase of the Human Menstrual Cycle

It is generally recognized that LH and FSH are synthesized together in the same gonadotropes, the amount of LH stored in the pituitary is greater than that for FSH, and there is a greater tonic or constitutive (GnRH-independent) secretion of FSH than that for LH[246] (see also Chapter 10). Regardless, to achieve differential release of the gonadotropins, as occurs during the late follicular phase of the human menstrual cycle, it would seem that LH and FSH must be differentially distributed in at least a cohort of granules/vesicles that are destined for exocytosis. Ultrastructure examination of the anterior pituitary of the ewe revealed that the gonadotropin immunopositive granules contained either LH only or LH and FSH in combination, with the LH-only cells predominating.[247]

The frequency of the GnRH pulse generator does not appear to change during the late follicular phase of the human menstrual cycle when circulating FSH levels are declining at a greater rate than those of LH (see above), and therefore the differential secretion of the gonadotropic hormones during this phase of the cycle must be achieved at the level of the anterior pituitary, as a result of either a decline in GnRH pulse amplitude and/or an increase in estradiol concentrations to which the gonadotropes are exposed. Since the relative changes in circulating FSH and LH levels during the latter half of the follicular phase in

women that are hypophysiotropically clamped are essentially identical to those in normal women,[248] it would seem that any role of decreased amplitude of the intermittent GnRH input to the pituitary is dispensable. This view is consistent with other findings from hypophysiotropically clamped women. Marshall et al.[226] found that estradiol administration to hypogonadotropic women, which resulted in follicular phase levels of the circulating steroid, profoundly suppressed the FSH response to invariant pulsatile GnRH stimulation with lesser suppression of LH. Moreover, in postmenopausal women in whom the pituitary was acutely emancipated from hypothalamic input by administration of a GnRH receptor antagonist (see above), chronic exposure to follicular phase levels of estradiol blunted the response of FSH to exogenous GnRH to a greater extent than that of LH.[194]

The question therefore becomes how is the negative feedback action of estradiol on gonadotropin secretion during the late follicular phase of the human menstrual cycle able to produce a suppression of FSH that is greater than that of LH? The most compelling hypothesis at the present time is that estradiol inhibits the activin tone within the pituitary and therefore reduces the paracrine drive for constitutive release of FSH. Activin B is the major activin in the pituitary and synthesis of this paracrine factor occurs in the gonadotropes themselves, which also express the repertoire of membrane receptors necessary for activin signaling (details of which may be found in Chapter 10). Support for the foregoing hypothesis has been provided by Nett and his colleagues, who demonstrated in primary culture of pituitary cells from the ewe that addition of physiological estradiol concentrations (0.01–1.0 nM) produced a dose-dependent suppression of activin β_B mRNA.[249] Interestingly, in the same study, estradiol did not influence the expression of follistatin, another pituitary factor that selectively modulates FSH secretion by its ability to bind to activin and abrogate its action.[250]

The reason for the difference in the extent to which the secretion of FSH and LH is differentially regulated during the late follicular phase of the human and monkey menstrual cycle is unclear, but it is conceivable that the characteristics of the assays used to measure monkey and human gonadotropin levels in blood may contribute to this apparent species difference. Regardless, in both women and the female monkey the decline in FSH secretion produced by the negative feedback action of increasing estradiol production by the follicle destined to ovulate is the critical component underlying the ovulation of a single follicle during the primate menstrual cycle.

Summary of Follicular Development and Clinical Implications

The selection of the preovulatory follicle in primates is best explained by the concept of a critical "threshold" concentration of FSH that must be achieved to stimulate the development of early antral follicles to the preovulatory stage. As depicted in Figure 28.8, during the luteal phase of the menstrual cycle the corpus luteum restrains FSH secretion (by mechanisms to be discussed later). Upon the regression of the corpus luteum, plasma FSH concentrations rise and early antral follicles undergo FSH-dependent growth and differentiation. The number of follicles that develop to the preovulatory stage depends on the duration that FSH concentrations are maintained above threshold values. By way of the FSH-dependent production of estradiol, the leading follicle suppresses FSH secretion below the threshold and further maturation of follicles is prevented, whereas the maturing follicle continues to develop because the FSH-mediated induction of LH receptors on the maturing follicle protects it from its self-imposed reduction of FSH secretion.

The concept that continued maturation of the preovulatory follicle in the face of declining FSH levels is due,

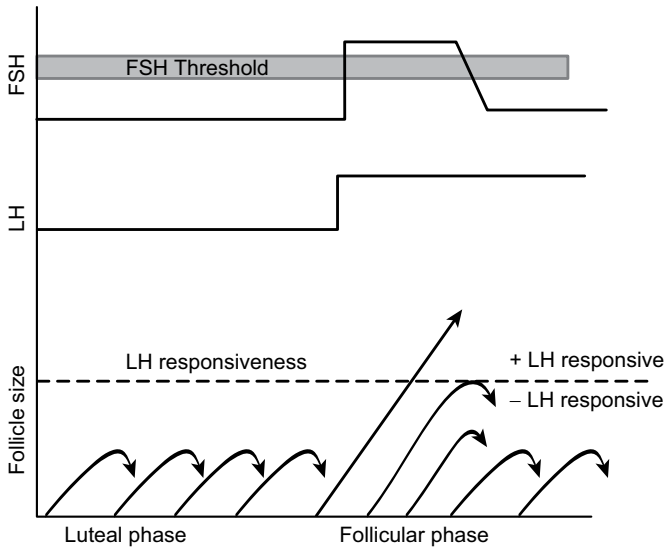

FIGURE 28.8 The follicle-stimulating hormone (FSH) threshold model for the selection of the preovulatory follicle. During the luteal phase of the menstrual cycle, circulating FSH concentrations are held below the FSH threshold by secretions of estrogen, progesterone, and inhibin by the corpus luteum and growing follicles do not advance beyond the preantral stage and undergo atresia. Upon the regression of the corpus luteum at the end of the menstrual cycle, the negative feedback restraint on FSH secretion is released and FSH concentrations rise above threshold levels. One (or occasionally more) of the maturing preantral follicles is stimulated in response to the elevation of FSH and develops both the P450AROM enzyme and luteinizing hormone (LH) receptors. The acquisition of P450AROM produces a rise in systemic levels of estradiol that results in the suppression of FSH secretion, which, in turn, prevents the maturation of less mature follicles. The FSH-stimulated induction of LH receptors and the acquisition of LH responsiveness of granulosa cells of the stimulated follicle permit the FSH-stimulated follicle to mature in the presence of FSH concentrations that are insufficient to stimulate the maturation of other less mature follicles that undergo atresia due to the lack of sufficient FSH stimulation.

at least in part, to its acquisition of LH responsiveness has led to novel paradigms for ovulation induction in humans.[251] Traditional ovulation induction protocols typically involve the administration of recombinant FSH or human menopausal gonadotropins to stimulate follicular growth to the preovulatory stage, whereupon human chorionic gonadotropin is given to induce final oocyte maturation. The inherent problem with this approach is that the prolonged administration of FSH throughout the entire course of treatment would result in the asynchronous recruitment and maturation of follicles (Figure 28.9). The administration of hCG to promote the final maturation of the oocytes may thus result in the recovery of suboptimal oocytes from less mature follicles that may have diminished developmental potential when compared with oocytes from more mature follicles. Further, the continuous administration of FSH may also result in the maturation of smaller follicles, which could lead to the development of ovarian hyperstimulation following the administration of hCG.[251]

However, switching from FSH to LH during the latter period of ovulation induction could result in the maturation of a more synchronous cohort of follicles and thereby produce a greater percentage of mature oocytes as well as reduce the number of smaller, less mature follicles and thereby reduce the incidence of ovarian hyperstimulation (Figure 28.8). Filicori and colleagues were the first to apply this strategy in the clinic.[252] Instead of using LH to support the final maturation of follicles, they administered a low dosage of hCG as a surrogate for LH to sustain the final maturation of FSH-stimulated preovulatory follicles. Their results indicated that switching from FSH to hCG did not reduce the number of large preovulatory follicles but did reduce the number of smaller developing follicles. Further, the fertilization rate of oocytes collected from these follicles was greater than that observed in women who received FSH alone, and pregnancy rates were comparable in both groups of patients. In a larger randomized control trial, Blockeel et al.[253] demonstrated that substitution of FSH by low-dose hCG in the final days of ovarian stimulation produced comparable yields of oocytes and pregnancy rates when compared with subjects that received FSH alone, although they were unable to confirm a reduction in small antral follicles in the hCG-treated group. However, as would be expected, both studies reported a significant reduction (approximate 25%) in the amount of FSH necessary to achieve successful ovarian stimulation, which would result in a substantial reduction in the cost of treatment.

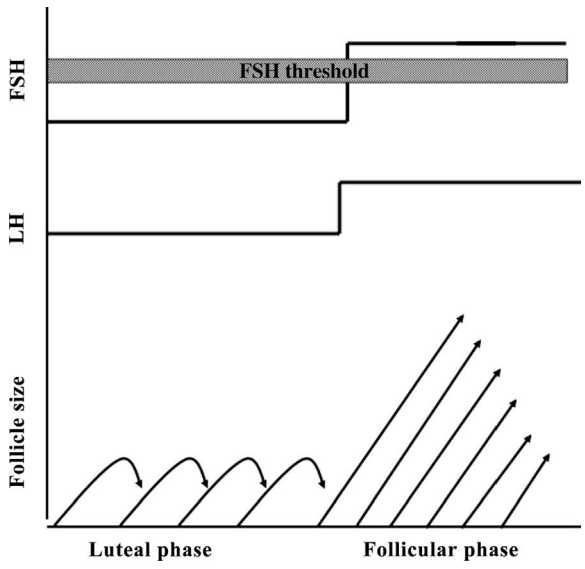

FIGURE 28.9 Conventional controlled ovarian stimulation protocol. Recombinant FSH or human menopausal gonadotropins are administered to elevate blood follicle-stimulating hormone (FSH) concentrations above the threshold level, which results in the stimulation of preovulatory follicular development. Prolonged treatment with FSH may result in asynchronous recruitment and maturation of follicles, which may lead to recovery of suboptimal oocytes from less mature follicles as well as increasing the number of smaller follicles, which could result in ovarian hyperstimulation.

OVULATION

The follicular phase ends with an abrupt rise in blood levels of LH and FSH (Figure 28.1). The role of the midcycle surge of LH in initiating the processes of ovulation and luteinization was first explored in primates by Moudgal et al. who demonstrated that injection of antiserum that neutralized circulating LH on days 10–13 of the menstrual cycle blocked ovulation and the expected rise in progesterone secretion in cynomolgus monkeys.[254] Weick et al.[255] performed daily laparotomies in rhesus monkeys beginning at the peak of estrogen levels and noted that the time of ovulation, as reflected by the presence of an ovulatory stigmata on the surface of the ovary as well as newly formed corpora luteum, occurred approximately 37 h after the estimated time of the LH peak. The ovarian events associated with the control of ovulation and oocyte maturation are described in detail in Chapters 2 and 22 and in a comprehensive review of the molecular control of ovulation and luteinization in primates.[256] The foregoing is a brief summary of the major factors that are thought to play a role in LH-induced ovulation in primates.

Epidermal Growth Factor-like Ligands

As described in detail in Chapter 22 and elsewhere,[257] studies in rodents have shown that the ovulatory surge of LH, acting through its receptors on mural granulosa cells, stimulates the production of the epidermal growth factor (EGF)-like proteins amphiregulin, epiregulin, and betacellulin, and that these ligands mediate the actions

of LH in the process of cumulus-oocyte expansion, resumption of meiosis of the oocyte, prostaglandin synthesis, and expression of proteolytic enzymes involved in follicle rupture. In monkeys and humans, amphiregulin (AREG) appears to be the primary EGF-like ligand stimulated by an ovulatory gonadotropin stimulus and is able to stimulate the cumulus expansion and resumption of oocyte meiosis.[258–260] The extent to which AREG mediates other ovulatory effects of LH remains to be established.

Prostaglandins

A role for prostaglandins in the process of ovulation in primates was identified by Wallach et al.[261] who demonstrated that the prostaglandin synthesis inhibitor indomethacin was capable of blocking gonadotropin-induced (hMG + hCG) ovulation, as assessed by laparotomy, in the rhesus monkey. Similarly, ultrasonic scanning demonstrated that inhibition of prostaglandin synthesis resulted in luteinized unruptured follicles in humans.[262] Studies by Duffy and Stouffer demonstrated that an ovulatory hCG stimulus produced an elevation in cyclooxygenase-2 and prostaglandins E_2 and $F_{2\alpha}$ in the ovulatory follicle.[263] A local role for prostaglandins in the process of ovulation was confirmed by the demonstration that an intrafollicular injection of indomethacin in rhesus monkeys blocked ovulation but did not inhibit the process of luteinization as reflected by a normal pattern of progesterone levels during the posttreatment luteal phase and histological identification of luteinizing granulosa cells. Simultaneous intrafollicular injection of indomethacin plus prostaglandin E_2 restored ovulation.[264] Although the evidence that prostaglandins are required for ovulation in primates is strong, the precise mechanisms by which prostaglandins govern the process of ovulation remain to be identified. Recent studies[265] have identified the distribution of PGE_2 receptor subtypes in the macaque ovulatory follicle and found that the PGE_2 receptor EP1 was stimulated in mural granulosa cells following hCG-induced ovulation, while EP2 and EP3 were present in cumulus cells to a greater extent than mural granulosa cells following hCG, and EP4 levels were uniformly low in all granulosa cell subtypes. These results are consistent with the notion that the EP1 receptor regulates follicle rupture by way of its stimulation of proteolytic enzymes, while the EP2 and 3 receptor subtypes are involved in cumulus cell expansion.

Progesterone

Early studies in rodents demonstrated that inhibition of steroid biosynthesis blocked ovulation and that supplementation with progesterone restored the ovulatory response.[266] Moreover, as discussed in detail in Chapter 22, genetically modified mice that lack the progesterone receptor (PR) fail to ovulate.[267] A role for progesterone in the process of ovulation in primates was identified by the observation that systemic treatment of rhesus monkeys with the steroid biosynthesis inhibitor trilostane blocked gonadotropin-stimulated ovulation and oocyte maturation and that this inhibition was reversed by coadministration of the progestin agonist R5020.[268] The mechanisms by which progesterone participates in ovulation may involve, at least in part, the stimulation of a variety of proteolytic enzymes involved in ovulation (see below).

Proteolytic Enzymes

The classic studies of Rondell[269] demonstrated in rabbits that, contrary to expectations, ovulation was not an explosive event due to increased intrafollicular hydrostatic pressure but rather to a weakening of the follicle wall as a result of proteolytic degradation of the extracellular matrix. He further demonstrated that the production of progesterone was essential for the increased distensibility (weakening) of the follicle wall and proposed that, in response to the LH surge, progesterone activates an enzymatic cascade that leads to weakening and rupture of the follicle wall. A number of proteolytic enzymes, including matrix metalloproteinases (MMPs) disintegrin and metalloproteinases with thrombospondin-like repeats (ADAMTSs) and plasminogen activators (PAs), involved in tissue remodeling are induced by the ovulatory stimulus in monkeys and ovulation can be blocked by the intrafollicular injection of a metalloproteinase inhibitor.[270,271] As noted before, one role of progesterone in the ovulatory process appears to be related to the regulation of these proteolytic enzymes as inhibition of steroidogenesis blocks ovulation and the expression of ovulation-associated proteolytic enzymes.[270]

Neuroendocrine Control of the Mid-cycle Gonadotropin Surge

The preovulatory gonadotropin surge is initiated by the positive feedback action of the rising levels of circulating estradiol that are produced as a result of increased secretion of the steroid by the follicle that is destined to ovulate. For estradiol to exert a positive feedback action on pituitary gonadotropin secretion it must be maintained above a threshold serum concentration for a duration of several hours. In the monkey, Knobil and his colleagues systematically varied these two parameters of the circulating estradiol signal by the administration of square wave increments of exogenous steroid during the early follicular phase: a premature LH surge was only elicited when an estradiol threshold of 200–400 pg/ml was maintained for a minimum of 36 h.[45]

This relationship was termed the strength-duration characteristic of the positive feedback action of estradiol.

Small quantities of progesterone are secreted prior to ovulation by the ovary in both women and female monkeys,[255,272,273] and this preovulatory release of the steroid has been implicated in the genesis of the LH surge. However, the findings in both species that LH surges may be prematurely elicited in the early follicular phase[45,46,274,275] and triggered in ovariectomized monkeys by administration of estradiol alone[50] has led to the view that progesterone's role is limited to amplifying the magnitude of the gonadotropin surge that is initiated by the positive feedback action of estradiol. This action of progesterone and other stimulatory effects of the steroid on gonadotropin secretion were discussed in full in the previous edition of this book.

As in the case of the negative feedback action of estradiol on pituitary gonadotropin secretion, the site of the positive feedback of the steroid may also be exerted directly at the pituitary, or indirectly at the level of the hypothalamus, to increase GnRH stimulation of the gonadotropes or at both these sites. The finding with hypophysiotropically clamped models employing GnRH-deficient women and hypothalamic-lesioned female monkeys that the preovulatory gonadotropin surge and ovulation were restored when the pituitary was stimulated by invariant GnRH administration (Figure 28.6) provides convincing evidence that positive feedback of estradiol solely at the pituitary is sufficient to elicit a functional LH surge.[223–225,233] The results obtained from the human models are particular compelling for two reasons. First, the pulse doses of GnRH selected to drive the pituitary–ovarian axis were in many cases chosen because they induced episodes of LH secretion that mimicked those observed spontaneously in normal women. Second, subjects with Kallman syndrome, when employed, guaranteed that the hypothalamic GnRH deficiency was absolute as migration of GnRH during embryonic development in these patients is arrested in the forebrain.[276] During normal embryonic development in primates and other species, GnRH neurons migrate from the olfactory placode through the forebrain to the hypothalamus and in the adult brain are found in relatively large numbers in both the rostral hypothalamus and more caudal regions including the MBH (see Chapter 11).

During spontaneous menstrual cycles in the monkey, however, a positive feedback action of estradiol is also evident at the hypothalamus, as demonstrated by the finding of Spies and his colleagues that the preovulatory LH surge in this primate is associated with a corresponding and unequivocal discharge of GnRH, as measured in perfusates of the median eminence collected during the LH surge.[277] The heightened release of GnRH at the time of the mid-cycle LH surge in the monkey does not appear to be triggered by an action of estradiol on the GnRH pulse generator, since hypothalamic MUA appears to be arrested during the spontaneous preovulatory LH surge in this species[53] (Figure 28.7). Thus, it becomes necessary to posit a GnRH surge generator in the monkey hypothalamus, as is the case for nonprimate species (see Chapters 26 and 27). In contrast to the rodent, however, the LH surge in the monkey is not tightly coupled to the circadian system, as indicated by the finding that the timing of the LH surge may be advanced by up to 12 h when the strength of the estradiol positive feedback signal is increased to a supraphysiological level.[45]

Whether a GnRH surge generator is resident in the human hypothalamus is unclear. In contrast to the monkey, GnRH pulse generator activity, as reflected by pulsatile patterns of circulating LH, continues unabated during the spontaneous preovulatory gonadotropin surge in women.[278] In postmenopausal women, metabolic activity in the MBH and pituitary as determined by monitoring [^{18}F]2-fluoro-2-deoxy-D-glucose uptake using positron emission tomography was increased only in the pituitary during an estradiol-induced LH surge.[279] Moreover, Hall and her colleagues have argued that the amount of GnRH secreted at this stage of the human menstrual cycle is actually decreased[280]: a view based on the finding that the amount of GnRH receptor antagonist required to effect a particular level of LH and FSH suppression during the mid-cycle surge is less than that needed to effect the same level of suppression during the follicular phase. This observation also indicates that the pituitary gonadotrope becomes more sensitive to GnRH during the LH surge, as would be predicted by the pituitary being a primary site of action for the positive feedback of estradiol (see above). The spontaneous initiation of the preovulatory LH discharge in the human female generally occurs in the morning[281–283] in association with high cortisol levels.[283] This temporal relationship, however, appears to be independent of an endogenous circadian rhythm, as recently demonstrated by the finding that amplitude and frequency of pulsatile LH secretion do not vary over a 24 h period in premenopausal women studied under a rigidly controlled environment of constant lighting, activity, posture, wakefulness, and nutritional intake.[284]

While the GnRH surge generator in the rodent is located in the preoptic area (POA) of the rostral hypothalamus (see Chapter 26), the situation in the monkey is less clear. A series of studies by Knobil's group in the 1970s led to the proposal that the neuroendocrine mechanisms governing the LH surge in the monkey were resident entirely within the MBH. Application to the monkey of the Halasz knife (a bayonet-shaped knife that may be lowered to the base of the brain using stereotaxic and imaging techniques) to surgically isolate the MBH from the rest of the brain, including more anterior aspects of the hypothalamus such as the POA, and placement of large lesions in the POA that destroyed the suprachiasmatic nucleus neither abolished ovulatory menstrual cycles nor blocked estradiol-induced LH surges.[55,285]

In addition, estradiol-induced LH surges in ovariectomized monkeys were observed in an acute decerebrate preparation, in which all hypothalamic tissue dorsal to the optic chiasm had been removed by aspiration.[286] These findings[a] indicate that in the monkey the hypothalamic GnRH surge generator (like the pulse generator) is localized in the MBH.

The classic view of the neuroendocrine mechanism underlying the preovulatory LH surge in the monkey, which is based largely on studies by Knobil's laboratory more than 30 years ago, merits reexamination in light of the recognition of the critical importance of kisspeptin neurons in regulating GnRH release. This is for two reasons: first, in the rodent, the positive feedback action of estradiol, that together with a circadian signal from the suprachiasmatic nucleus trigger the preovulatory GnRH surge in these species, is mediated, at least in part, by activation of kisspeptin neurons of the AVPV in the POA (see Chapters 26 and 27); and second, although neuroanatomical studies of the primate hypothalamus are limited, in both female monkeys and women *KISS1* and kisspeptin neurons are also located in the POA[58,77,288] in areas that are likely to be analogous to the AVPV of rodents. Moreover, high levels of *KISS1* expression in the POA of the female monkey have been reported using quantitative real-time-PCR (qRT-PCR).[289] The regulation of *KISS1* expression by estradiol in the primate POA, however, has only been scantly studied. In contrast to rodents, *KISS1* mRNA levels in the POA of the monkey as determined by qRT-PCR did not decrease following ovariectomy.[289] However, an increase in *KISS1* mRNA/neuron was observed by in situ hybridization in the POA of female monkeys during the late follicular phase in association with elevated blood estradiol levels, and interestingly this was accompanied by a similar, albeit less marked, change in *KISS1* expression in the caudal third of the arcuate nucleus.[288] This finding led Smith et al. to propose that in the monkey both populations of kisspeptin neurons may be involved in mediating the positive feedback action of estradiol on gonadotropin secretion.[288] Such a conclusion may be reconciled with that of Knobil by proposing that the kisspeptin neurons in the POA of the monkey hypothalamus are not essential for the positive feedback action of estradiol and that the MBH-pituitary unit is sufficient for the generation of functional preovulatory LH surges (Figure 28.10). Moreover, if the proposal of Smith et al. is substantiated, it would seem reasonable to further propose that the arcuate nucleus of the monkey must contain two distinct populations of kisspeptin neurons: one involved in GnRH pulse generation and in mediating the negative feedback action of estradiol, and the other in transducing the positive feedback action of the steroid. Presumably, the activity of the first population as reflected by MUA activity will be inhibited during the positive feedback action of estradiol,[53] while that of the second population will be increased by an estradiol signal that exceeds the strength-duration characteristic necessary for induction of the LH surge. An alternative possibility is that the same neurons respond differentially to low (negative feedback) and high (positive feedback) levels of circulating estradiol.

Neural systems other than those comprised of kisspeptin neurons in the POA and arcuate nucleus have also been implicated in the modulation of the LH surge

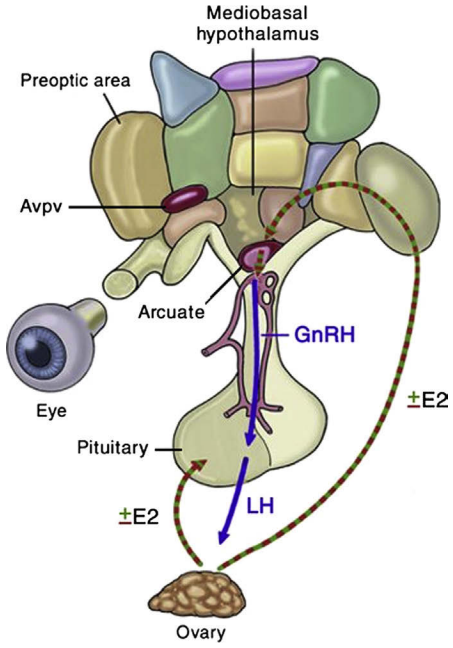

FIGURE 28.10 A model of the neuroendocrine mechanisms underlying the positive and negative feedback actions of ovarian estradiol (E2) on luteinizing hormone (LH) secretion during the menstrual cycle. In this model, the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator is located in the arcuate nucleus, which contains one of the two major hypothalamic populations of kisspeptin neurons (the second population of kisspeptin neurons is found in the anteroventroperiventricular nucleus (AVPV) in the preoptic area). The negative feedback action of E2 that regulates tonic LH secretion during the follicular phase and luteal phase (in combination with progesterone) is exerted on the GnRH pulse generator to primarily suppress GnRH pulse amplitude and on the pituitary to inhibit responsiveness to pulsatile GnRH stimulation. In the monkey, the positive feedback action of E2, which triggers the preovulatory LH surge, is also exerted within the medial basal hypothalamus (MBH) and at the pituitary. In the human female, the significance of the MBH in mediating the positive feedback action of estradiol is less clear: in both species positive and negative feedback actions of E2 solely at the pituitary level are sufficient for menstrual cyclicity and ovulation.

[a] In a contemporaneous study by Spies and his colleagues, anterior hypothalamic lesions were reported to block spontaneous and estrogen-induced gonadotropin surges, and this group concluded that the POA was necessary for induction of the preovulatory LH surge in the monkey.[287]

in primates. These have included the brainstem norepinephrine neurons,[290] which, as may be expected from their location, appear to play a nonessential role[50] (also see Chapter 33), and the MBH and pituitary tachykinin, substance P.[291,292] However, since 2003 the neuroendocrine community studying the hypothalamic control of the LH surge has become kisspeptinocentric as a result of the signal discovery in that year that loss of function mutations in *KISS1R* in man were associated with hypogonadotropic hypogonadism and delayed or absent puberty,[42,43] and few attempts have been made to incorporate other neurotransmitters/neuropeptides into contemporary models for generation of the LH surge.

While the precise neurophysiologic mechanisms underlying the spontaneous generation of the LH surge late in the follicular phase in primates remain to be fully delineated, the extant data permit several inferences/conclusions to be drawn. First, as may be anticipated from a process as important as ovulation, major redundancy is found in the control system that generates the preovulatory LH surge, the proximate trigger for ovulatory process. In this regard, the minimal hypothalamic input that is required for the surge to be generated is the pulsatile secretion of GnRH that permits, in turn, the positive feedback action of estradiol to be expressed at the pituitary. Under physiological conditions, however, an additional hypothalamic mode of GnRH release, not reflected by MUA, is engaged, at least in the monkey. Second, the control of the preovulatory LH surge in primates, and therefore ovulation in these species, has become relatively emancipated from regulation by the POA of the hypothalamus, which in the rodent dictates that ovulation is rigidly coupled to the light–dark cycle. A full discussion of differences in the neural control of the preovulatory LH surge in rodents and primates falls beyond the scope of this chapter, but this subject has been recently reviewed by one of us.[293]

CORPUS LUTEUM FORMATION AND FUNCTION

After years of investigation of the function and regulation of the corpus luteum, George W. Corner commented, "These yellow bodies of the ovary have been puzzling to scientists ever since they were first described by de Graaf."[3] In retrospect, this statement is particularly accurate regarding the quest for the understanding of the physiological regulation of this gland in mammals because the mechanisms that individual species have evolved to control the function of the corpus luteum exhibit marked variations[294] (and see Chapter 23). It appears, at least on the surface, that higher primates have developed a simpler solution to the regulation of the corpus luteum than most other species. As in other mammals, the primate corpus luteum plays a pivotal role in the control of reproduction and, in doing so, its functions must be regulated accordingly. In nonfertile menstrual cycles, the corpus luteum has a finite 14- to 16-day life span after which its secretions of steroid and protein hormones cease and the tissue is eventually removed from the ovary. The regression of the corpus luteum in the absence of conception is obligatory for the initiation of a new menstrual cycle because its secretions of estradiol, progesterone, and possibly inhibin A exert negative feedback effects at the hypothalamic-pituitary axis that prevents the rises in FSH and LH secretion that are essential for the development of a new preovulatory follicle.[170,173] On the other hand, during menstrual cycles in which conception occurs and implantation ensues, the prolongation of luteal function beyond its usual life span is obligatory for the maintenance of pregnancy until the placenta assumes the primary responsibility for the production of steroid hormones.[295,296] An explanation of luteal function must therefore account for the mechanisms by which the functions of the corpus luteum are governed to accommodate its dual responsibilities.

Development of the Corpus Luteum

Preovulatory Determinants of Luteal Function

The corpus luteum is formed upon the culmination of preovulatory follicular development and the rupture of the mature Graafian follicle. As shown in Figure 28.1, the mid-cycle gonadotropin surge marks the transition point at which the secretion of follicular estradiol is replaced as the major ovarian steroid by the production of progesterone by the corpus luteum. Although the corpus luteum is not formed until after ovulation, developmental events that transpire during the follicular phase under the control of FSH are essential for the appropriate functioning of the corpus luteum. Granulosa cells isolated from immature follicles are steroidogenically quiescent and require stimulation by FSH in vitro before the secretion of estradiol and progesterone becomes evident. By contrast, granulosa cells collected from preovulatory follicles of humans and subhuman primates are capable of producing both estradiol and progesterone immediately after isolation.[104,297] This FSH-dependent differentiation of the granulosa cell is responsible not only for the production of estradiol by the maturing follicle but also for the preparation of the granulosa–lutein cells to rapidly initiate progesterone secretion after ovulation. As noted above, studies in subprimate species have shown that FSH stimulation of granulosa cells during preovulatory follicular development is associated with increases in the cellular content of mRNAs and/or enzyme activities of P450scc, 3β-hydroxysteroid

dehydrogenase, Δ^{5-4} isomerase (3β-HSD), P450AROM, and the LH receptor, all of which are required for the secretion of steroid hormones by the corpus luteum. The importance of FSH on luteal function by way of its effects on the differentiation of the follicular granulosa cell was shown by in vivo studies in subhuman primates that demonstrated a disturbance in the normal pattern of FSH secretion during the follicular phase, resulting in abnormal luteal phases characterized by reduced progesterone production.[298,299]

Luteinization

Although the granulosa cells of preovulatory follicles manifest LH-responsive estradiol and progesterone secretion that is characteristic of luteal cells, the transformation of the granulosa cell into a luteal cell by the mid-cycle LH surge is associated with a further accretion of the steroidogenic capacity of the cell and a further increase in the steady state levels of mRNAs for enzymes involved in progesterone production. This increased steroidogenic capacity occurs in parallel with changes in the morphology of the granulosa–lutein cells. In the rhesus monkey, granulosa cells from preovulatory follicles have a diameter of approximately 10 μm, contain sparse amounts of endoplasmic reticulum, and possess mitochondria with lamelliform cristae. By contrast, luteal cells of newly formed corpora lutea measure approximately 25 μm in diameter and possess abundant smooth and rough endoplasmic reticulum and mitochondria with tubular cristae.[300] The structural remodeling of granulosa cells during luteinization is dependent on high levels of LH because granulosa cells collected from rhesus monkey follicles before the LH surge fail to produce substantial amounts of progesterone in vitro and fail to exhibit morphological signs of luteinization.[301] In addition to alterations in cellular morphology, the process of luteinization is associated with terminal cellular differentiation. Studies in rats have convincingly demonstrated that corpora lutea do not incorporate [^{3}H] thymidine into nuclei, a measure of DNA synthesis, whereas such incorporation is readily apparent in follicular granulosa cells.[302]

Although comparable in vivo labeling studies have not been performed in primates, analysis of rhesus monkey ovaries has indicated that the expression of markers of proliferation such as Ki67 and proliferating cell nuclear antigen are absent in the parenchymal cells of the corpus luteum.[303,304] The molecular mechanisms by which cellular proliferation is curtailed in the corpus luteum is not completely understood, although it is now known that luteinization is associated with the reduced expression of cellular proteins thought to be involved in the regulation of cell cycle dynamics[305] (and Chapter 23). The luteinizing stimulus generated by the LH surge thus results in a coordinated series of responses of granulosa cells that include intracellular remodeling, enhanced gene expression, and modulation of the cell cycle. Although the precise cellular mechanisms by which LH initiates the coordinated processes of luteinization is not known, it is likely that the large increase in cAMP or other intracellular signaling molecules triggered by the ovulatory LH surge either activates or represses signaling pathways that control cellular differentiation and proliferation[306] (and Chapter 20). The morphological events associated with luteinization are not completed until 5–6 days after ovulation; thereafter, the luteal cells exhibit all the cellular characteristics associated with steroid-producing cells.[307]

Angiogenesis

The granulosa cell layer of preovulatory follicles is not vascularized; the capillary network that supplies the follicle abruptly terminates at the basement membrane that separates the granulosa cells from the theca cells. As described by Corner,[3] after ovulation, as the luteinizing granulosa layer grows thicker, blood vessels creep in from the theca layer and form a network that supplies every one of the large luteal cells. Within a few days after ovulation, the corpus luteum becomes highly vascularized such that on a tissue weight basis, blood flow to this gland is among the greatest of any tissue in the body.[308] This increased vascularity, in addition to providing a conduit for the delivery of luteal steroids to the general circulation, is also necessary for the provision of cholesterol substrate in the form of low-density lipoproteins (LDLs) for progesterone biosynthesis.[309]

The incisive experiments of Gospodarowicz and Thakral[310] demonstrated that the newly ovulated follicle produces diffusible angiogenic substances that direct capillary proliferation into the luteinizing tissue. As described above, mRNA for the angiogenic factor VEGF is expressed intensely by the maturing follicle, and this intense expression continues after ovulation such that the newly formed corpus luteum also expresses mRNA for VEGF[154] (and Chapter 23). The expression of mRNA for VEGF in the corpus luteum, as revealed by northern analysis, correlates with luteal function in that it is extinguished during spontaneous luteal regression and declines prematurely after suppression of LH secretion by a GnRH antagonist, and stimulation of luteal function by administration of CG extends the expression of mRNA for VEGF beyond the expected time of luteal regression.[220] The localization of mRNA for VEGF to structures within the primate ovary in which angiogenesis plays an important role in development is consistent with the hypothesis that the VEGF family of angiogenic factors may be instrumental in the formation of capillaries that accompanies both the development of the preovulatory follicle and the neovascularization that

is essential for the functioning of the corpus luteum. Indeed, studies in nonhuman primates have shown that administration of VEGF antagonists at the time of ovulation or during the luteal phase of the menstrual cycle attenuates progesterone production by the corpus luteum.[311,312] Additional discussion of the complex process of angiogenesis that occurs during luteinization and its maintenance throughout the lifespan of the corpus luteum is presented in Chapter 23.

Endocrine Secretions of the Corpus Luteum

Steroid Hormones

The description of the corpus luteum as an endocrine gland is based on its capacity to secrete progesterone and estradiol.[3,313] The production of progesterone and estradiol by the primate corpus luteum likely is regulated by separate steroidogenic pathways. As described above, the secretion of estradiol by the developing follicle requires the participation of both the theca cells for the production of androgen substrates and the granulosa cells for the aromatization of androgens to estradiol. Although the basement membrane that separates theca cells from granulosa cells is dissolved after ovulation, the primate corpus luteum retains some of the structural organization that was present in the preovulatory follicle. Using specific antibodies to steroidogenic enzymes, it has been shown that P450c17, a theca cell marker, is confined to the outer borders of the corpus luteum, whereas P450AROM, a granulosa cell marker, is present within the central area of the corpus luteum.[111] Assuming that the "two-cell" model for estradiol secretion by the maturing follicle can be extended to the corpus luteum, the production of estradiol therefore would require the coordinated activity of both theca lutein and granulosa–lutein cells.

Progesterone production by the corpus luteum is dependent on cholesterol as a substrate. Although luteal cells can manufacture cholesterol de novo from acetate, as mentioned above, the corpus luteum relies extensively on serum LDL-associated cholesterol for use as a precursor for the synthesis of progesterone.[314,315] The dependence of normal luteal secretion of progesterone on LDL cholesterol is reinforced by the finding that progesterone concentrations during the luteal phase of the menstrual cycle in a woman with hypobetalipoproteinemia, a genetic disorder characterized by low plasma concentrations of LDL, were nearly ten-fold lower than those of normal subjects.[316] The control of luteal steroidogenesis is described in greater detail in Chapters 8 and 23.

Protein Hormones

In addition to steroid hormones, the primate corpus luteum also synthesizes and releases a variety of protein hormones, including relaxin,[317–319] oxytocin[320–322] and inhibin.[323–325] Enucleation of the corpus luteum of cynomolgus monkeys resulted in a rapid decline in serum inhibin concentrations, indicating that most inhibin in blood is of luteal cell origin.[326] The secretion of inhibin throughout the luteal phase appears to reflect a unique function of the primate corpus luteum because neither inhibin A nor inhibin B appears to be produced to any great extent by corpora lutea of rodents and domestic animals.[327–330]

Control of Luteal Function and Life Span

Luteotropic Hormones

LUTEINIZING HORMONE

As discussed in the section on follicular maturation, the early to mid-1900s was a period in which the nature and biological actions of the pituitary gonadotropins were revealed. The parallel studies on the identification and purification of prolactin and the demonstration that this hormone had stimulatory effects on the corpus luteum of rodents[331,332] confounded the identification of the anterior hypophysial hormone responsible for the regulation of the primate corpus luteum. Hisaw,[333] in his pioneering studies on the regulation of the rhesus corpus luteum, proposed that prolactin was the primary luteotropin responsible for the maintenance of the corpus luteum of the menstrual cycle, whereas CG was required to extend the functional life span of the corpus luteum of pregnancy. Although it was well established by the early 1960s that LH-stimulated progesterone production by human corpora lutea in vitro,[334,335] not until the early 1970s was it shown that LH acutely increased progesterone production by the corpus luteum in vivo.[336,337]

The requirement for LH in the control of progesterone secretion and the functional life span of the corpus luteum has been the subject of numerous debates. Gemzell[29] noted that induction of ovulation by hCG in hypophysectomized women who had been treated with FSH to induce follicular growth was followed by luteal phases of normal duration, suggesting that once formed, the human corpus luteum functioned independently of additional pituitary gonadotropin support. Subsequent studies by Vande Wiele et al.[30] demonstrated that the long half-life of hCG used to induce ovulation provided extended gonadotropic support to the corpus luteum; when LH was used to induce ovulation, the corpus luteum regressed after 5–6 days unless additional LH was given throughout the luteal phase. An obligatory requirement for LH in the maintenance of the corpus luteum was indicated when Moudgal et al.[254] passively immunized rhesus monkeys with antibodies against LH and found that immunized animals exhibited premature luteal regression, an observation later confirmed by others.[338]

The role of LH in the maintenance of the primate corpus luteum was subsequently questioned by the

observations that neither hypophysectomy nor treatment with a GnRH antagonist to suppress LH secretion truncated the luteal phase of macaques.[339,340] However, studies in rhesus monkeys in which pituitary gonadotropin secretion was controlled directly by pulsatile infusion of synthetic GnRH provided compelling evidence that pituitary secretion of gonadotropins absolutely is required for luteal function.[341] As first described by Knobil and colleagues[56] and shown in Figure 28.11 (top), rhesus monkeys rendered anovulatory by the placement of lesions in the MBH in which menstrual cycles are restored by hourly intravenous pulses of GnRH have luteal phases of normal 14- to 16-day duration. As shown in Figure 28.11 (bottom), when gonadotropin secretion was interrupted in this experimental model by terminating the GnRH infusions, a rapid and precipitous fall in serum progesterone concentrations occurred and premature menstruation ensued in all animals. The initial report[340] that GnRH antagonists fail to shorten the life span of the corpus luteum appears to be dose related because others have shown in both macaques and humans that GnRH antagonists rapidly suppress luteal progesterone secretion and concomitantly evoke premature menses.[342,343]

In addition to the secretion of progesterone, LH also is required for the production of estradiol and inhibin by the corpus luteum as plasma concentrations of both hormones decline after treatment with GnRH antagonists.[342] Further, plasma concentrations of estradiol, inhibin, and relaxin are elevated during early pregnancy and in nonpregnant subjects in response to the administration of exogenous CG, suggesting that synthesis of these hormones is also gonadotropin regulated.[344–349]

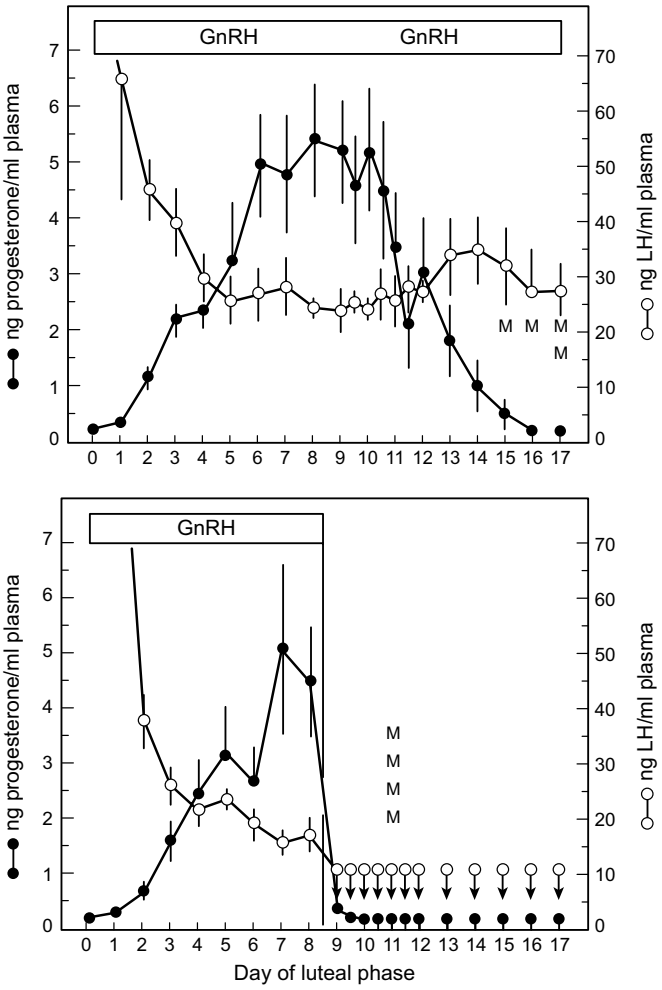

FIGURE 28.11 Serum luteinizing hormone (LH) and progesterone concentrations in medial basal hypothalamus (MBH)-lesioned rhesus monkeys whose menstrual cycles were restored by a pulsatile infusion of synthetic gonadotropin-releasing hormone (GnRH). The top panel illustrates serum LH and progesterone concentrations of animals that received GnRH at a frequency of 1 pulse/h throughout the menstrual cycle. Note that these animals have luteal phases of normal duration. The bottom panel illustrates data from animals in which the infusions of GnRH were terminated on day 8 of the luteal phase. Serum LH and progesterone concentrations fell rapidly upon the cessation of GnRH treatment and premature menses were observed in all animals. *Source: Reproduced with permission from Ref. 341, Copyright 1984, The Endocrine Society.*

FOLLICLE-STIMULATING HORMONE

Both FSH and LH secretion are reduced by GnRH antagonists, and the withdrawal of GnRH support to MBH-lesioned animals curtails both FSH and LH secretion.[350,351] Thus, experiments using these approaches to suppress LH secretion do not rule out the possibility that FSH may also participate in the regulation of the primate corpus luteum. FSH receptors have been identified in human corpora lutea,[352] but FSH does not stimulate adenylate cyclase activity in membranes prepared from macaque corpora lutea[353] and does not stimulate progesterone secretion in either human or macaque corpora lutea in vitro.[354,355] Although FSH has been shown to cause a slight (25%) increase in estradiol production by luteal cells isolated from human corpora lutea, this effect was observed in response to pharmacological concentrations of FSH,[356] and the possibility that the stimulatory effects of supraphysiological concentrations of FSH on luteal cell steroidogenesis are not a consequence of slight LH contamination of the FSH preparation cannot be ruled out. Further, the observations that exogenous hCG or LH alone is able to maintain luteal function in hypophysectomized women indicate that FSH does not play an obligatory role in the regulation of the primate corpus luteum in vivo.[29,30]

PROLACTIN

Prolactin is a principal luteotropin in rats and mice.[357] Whether this hormone exerts luteotropic effects on the primate corpus luteum is less clear, although it is known that the monkey corpus luteum expresses mRNA for the prolactin receptor.[358] Despite Hisaw's failure to extend the functional life span of the rhesus monkey corpus luteum with prolactin, he nonetheless concluded that

prolactin was the principal pituitary hormone responsible for the maintenance of lutea function until CG production by the implanting trophoblast commences.[333] Using the argument developed above against FSH as a luteotropin, the fact that normal luteal phases can be established in hypophysectomized women after FSH and hCG treatment argues against an obligatory requirement for prolactin in the maintenance of luteal function in primates.[29,30] Moreover, suppression of prolactin secretion during the luteal phase with a dopamine agonist does not interrupt normal luteal function in monkeys and humans.[359,360]

Although prolactin may not be an essential luteotropin in primates, elevated levels of prolactin may sustain luteal function beyond its typical life span of 14–16 days. Some rhesus monkeys rendered anovulatory by the placement of lesions in the MBH have elevated serum prolactin concentrations, presumably due to the destruction of the dopaminergic pathways that inhibit prolactin secretion. Although these hyperprolactinemic animals undergo ovulatory menstrual cycles in response to administration of exogenous GnRH, in some animals progesterone concentrations failed to decline to undetectable levels at the end of the luteal phase but rather were maintained at approximately 1 ng/ml for extended periods beyond the expected time of luteal regression.[361] That this extension of luteal function was due to prolactin was indicated by the findings that treatment of these hyperprolactinemic monkeys with the dopamine agonist bromocriptine resulted in a suppression in circulating prolactin concentrations and a prompt fall in serum progesterone levels. Similarly, lactation, which is associated with elevated prolactin concentrations, is known to stimulate progesterone secretion.[362] Treatment of suckling monkeys with bromocriptine suppressed prolactin secretion and reduced progesterone to undetectable levels.[361] Although the aforementioned results indicate that prolactin most likely does have stimulatory effects on the primate corpus luteum, it is unlikely that the corpus luteum is responsive to prolactin concentrations typically observed during the menstrual cycle, and that the responses of the corpus luteum during suckling and experimentally induced hyperprolactinemia reflects a response of the tissue to elevated concentrations of this hormone.

Autocrine and Paracrine Regulation of Luteal Function

STEROID HORMONES

The macaque and human corpus luteum contains PR, AR, and ERβ.[363–365] In vivo studies in monkeys have shown that blocking the production of progesterone with a 3β-HSD inhibitor shortens the luteal phase, suggesting that intraovarian progesterone may have an essential role in corpus luteum function.[366] Bishop et al.[367] used a microarray approach to identify mRNA transcripts that are regulated by LH and/or progesterone in the macaque corpus luteum. Results of this global analysis revealed that the greatest number of altered transcripts was observed when LH secretion was interrupted using a GnRH antagonist, while a smaller number of transcripts differed when progesterone biosynthesis was blocked with a 3β-HSD inhibitor. Of the approximate 1500 transcripts decreased by LH withdrawal, approximately 33% of these transcripts were also decreased by inhibition of steroid biosynthesis when LH levels were maintained by treatment of animals with exogenous LH. These findings indicate that whereas LH controls the vast majority of luteal transcripts, some of these LH-dependent genes are regulated indirectly by steroids produced by the corpus luteum. Exactly how LH and progesterone (or other steroids) interact to maintain luteal function remains to be established.

As described below, it has been proposed that estradiol may directly act at the level of the corpus luteum to promote luteal regression. There have been no systematic studies undertaken to identify a role of androgens on corpus luteum function, other than to serve as a substrate for estradiol biosynthesis by the corpus luteum.

PROTEINS

As noted above, although a number of proteins, including inhibin, activin, and IGFI, have been shown to influence steroid production by theca and granulosa cells of humans and subhuman primates, little information is available regarding the actions of these putative autocrine and paracrine agents on the primate corpus luteum. Studies have shown that activin reduced progesterone production by cultured macaque luteal cells, whereas inhibin had no effects.[368] In vivo, peripheral infusion of activin A suppressed progesterone production and shortened the luteal phase of rhesus monkeys.[369] However, in these studies, the infusion of activin also suppressed LH secretion. When activin was infused directly into the corpus luteum, neither progesterone production nor the length of the luteal phase was affected, indicating that the luteolytic effects of activin were most likely caused by a suppression of LH secretion rather than by a direct effect of activin on the corpus luteum.

Cellular Regulation of Luteal Function

The corpus luteum of nonfertile menstrual cycles has a finite 14- to 16-day life span during which time its functional presence is manifested by the secretion of progesterone and estradiol. Although serum progesterone concentrations are typically used to assess the functional status of the corpus luteum, studies in MBH-lesioned rhesus monkeys whose menstrual cycles are driven by pulsatile infusion of exogenous GnRH have shown that

the gonadotropic regulation of progesterone secretion can be dissociated from the control of luteal life span.[370] As shown in Figure 28.12, the termination of GnRH infusions in MBH-lesioned animals resulted in a rapid fall in serum progesterone concentrations, demonstrating that pituitary gonadotropin secretion is required for progesterone production by the corpus luteum. However, when LH secretion was restored 3 days later in these animals, serum progesterone secretion resumed and persisted for a time that effectively completed the duration of a typical luteal phase. Although the withdrawal of LH for a three-day period resulted in a suppression of progesterone secretion and premature menses, it did not compromise the functional life span of the corpus luteum. This suggests that although LH may be required for the acute stimulation of progesterone production, it may not be required for the long-term maintenance of the functional capacity of the corpus luteum. A complete investigation of the regulation of the primate corpus luteum must therefore address both the acute regulation of progesterone production and the long-term control of its steroidogenic capacity.

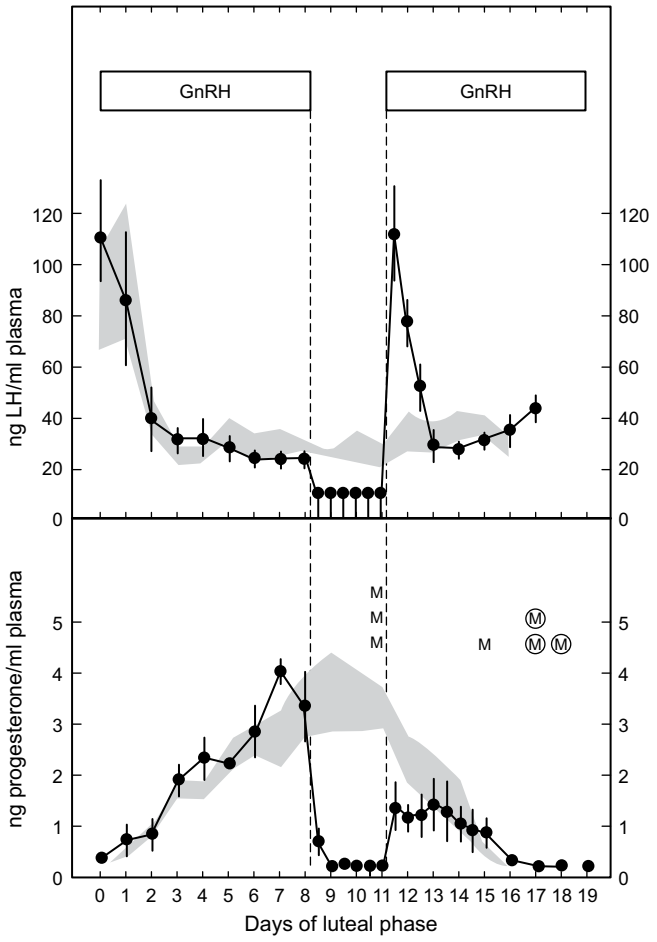

FIGURE 28.12 The primate corpus luteum is capable of recovering from a transient withdrawal of pituitary gonadotropin support. Medial basal hypothalamus (MBH)-lesioned rhesus monkeys whose menstrual cycles were driven by exogenous gonadotropin-releasing hormone (GnRH) were deprived of the GnRH infusion from days 8–11 of the luteal phase of the menstrual cycle. During the interval of deprivation of gonadotropins, serum progesterone concentrations fell to nondetectable levels and premature menstruations occurred. However, when luteinizing hormone (LH) secretion was restored by reinstating the GnRH infusions, progesterone production recommenced, and the corpus luteum regressed at its normal time, 16 days after ovulation. The shaded areas show data from animals in which GnRH was provided at a frequency of 1 pulse/h throughout the luteal phase. *Source: Reproduced with permission from Ref. 370, Copyright 1985, The Endocrine Society.*

Acute Actions of Gonadotropins

Studies in both macaques and humans have revealed a close temporal relationship between secretory pulses of LH by the pituitary gland and episodes of progesterone and estradiol production by the corpus luteum.[371,372] Elevations in plasma progesterone concentrations occur within minutes after individual pulses of LH. Because of the very rapid increases in steroid secretion in response to individual LH discharges, it is likely that the acute effects of LH on progesterone production by the corpus luteum reflect the channeling of cholesterol substrate through extant biosynthetic pathways rather than increases in the intracellular concentrations of enzymes responsible for progesterone synthesis. Addition of cAMP to dissociated luteal cells results in rapid increases in progesterone production,[373] indicating that the acute stimulatory effects of LH on luteal steroidogenesis are mediated by cAMP. The precise intracellular mechanisms responsible for the acute mobilization of cholesterol are unknown but likely include the intracellular transport of cholesterol and its access to P450scc at the inner mitochondrial membrane mediated by the steroidogenic acute regulatory protein, StAR[374] (and Chapter 8).

Long-term Actions of Gonadotropins

Although it is well established that LH is obligatory for the acute production of progesterone by the corpus luteum, the extent to which LH is involved in the longer-term regulation of the steroidogenic capacity of the corpus luteum has not been explored in great detail. The cloning of the genes for the enzymes involved in estradiol and progesterone biosynthesis has provided powerful tools for the investigation of luteal function at the cellular level.[375–378] Figure 28.13 illustrates serum progesterone concentrations and the cellular expression of mRNAs for P450scc and 3β-HSD in corpora lutea at defined stages of the luteal phase of cynomolgus monkeys.[379] Surprisingly, the highest concentrations of both mRNAs are seen shortly after ovulation. mRNA for P450scc remained relatively constant throughout the luteal phase until regression, whereas mRNA for 3β-HSD declined throughout the entire luteal phase. Similar findings were observed with human corpora lutea

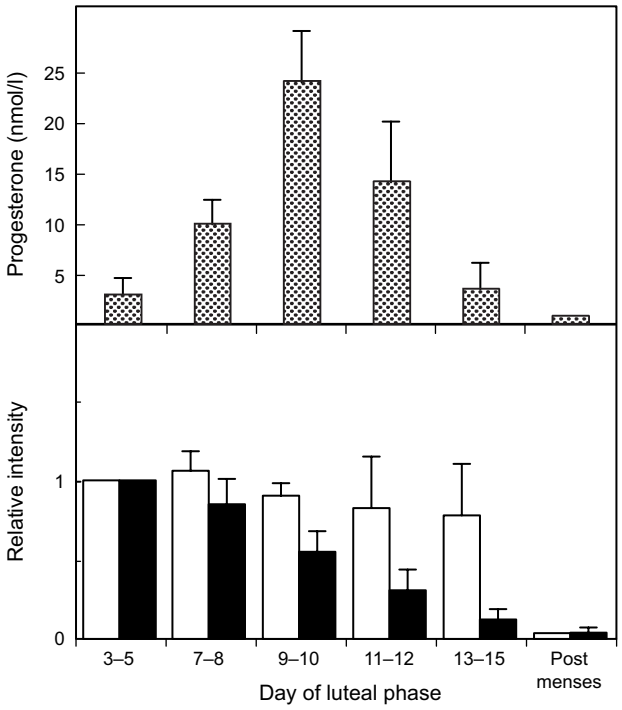

FIGURE 28.13 Expression of mRNAs for P 450scc and 3β-HSD throughout the luteal phase of cynomolgus monkeys. Corpora lutea were collected at defined stages of the luteal phase, as defined on the *x* axis. The top panel illustrates serum progesterone concentrations of the animals immediately before removal of the corpora lutea. The bottom panel illustrates levels of mRNAs for P450scc (open bars) and 3β-HSD (solid bars). *Source: Reproduced with permission from Ref. 379, Copyright 1991, The Endocrine Society.*

in that mRNAs for P450scc, 3β-HSD, and StAR were at their highest levels during the early luteal phase.[380,381] The progressive fall in these mRNAs throughout the luteal phase parallels the steroidogenic capacity of isolated luteal cells because it has been shown that luteal cells collected from newly formed human corpora lutea have greater basal and gonadotropin-stimulated progesterone production than cells obtained during the mid-luteal phase, when serum progesterone concentrations are maximal.[354,355] The discrepancy between progesterone biosynthetic capacity in vitro and the in vivo secretion of progesterone by the newly formed corpus luteum likely reflects the fact that the newly formed corpus luteum has yet to establish an extensive microvascular network, and therefore the delivery of precursor via LDL cholesterol to luteal cells is limited.[309]

The progressive decline in mRNAs for enzymes involved in steroid secretion and steroidogenic capacity of isolated luteal cells in vitro throughout the luteal phase suggests that the absolute steroidogenic capacity of the corpus luteum is set by the mid-cycle gonadotropin surge and declines thereafter independently of the overall pattern of gonadotropin secretion. A similar observation was reached in the investigation in the rat corpus luteum, which demonstrated that after luteinization, the expression of mRNA for P450scc does not require continued gonadotropin support.[382,383] However, the notion that LH does not directly participate in the long-term regulation of mRNAs for steroidogenic enzymes in the primate corpus luteum was dispelled when it was shown in cynomolgus monkeys that suppressing LH secretion during the luteal phase with a potent GnRH antagonist resulted in a decline in mRNA concentrations for P450scc and 3β-HSD to undetectable levels over a three-day period.[384] As noted above, global analysis gene expression profiles in the monkey corpus luteum following curtailment of LH secretion with a GnRH antagonist revealed that approximately 1500 transcripts were decreased following withdrawal of LH support.[367] Thus, LH, in addition to its obligatory role in the acute stimulation of progesterone production, is also required for the long-term maintenance of the functioning of the primate corpus luteum.

Regression of the Primate Corpus Luteum

Alteration of the Pattern of LH Secretion

Given that LH is required for both the acute and long-term regulation of luteal function, it would not be illogical to surmise that changes in LH secretion during the luteal phase could be responsible for the initiation of luteal regression. The most striking change in LH secretion during the luteal phase of both human and subhuman primates is the progesterone-mediated reduction of the frequency of LH pulses from approximately 1 pulse/h during the early to mid-luteal phases to 1 pulse/4–8 h from the mid through late luteal phases.[371,372] However, as originally documented by Knobil and colleagues[56] and later by others as shown in Figure 28.11, maintenance of menstrual cycles in anovulatory rhesus monkeys and humans by unvarying GnRH pulse frequencies typical of the follicular and early luteal phases does not prolong the life span of the primate corpus luteum.[385–387] Moreover, as shown in Figure 28.14, when an LH pulse frequency of one pulse per eight hours, which is typical of the mid through late luteal phases of the menstrual cycle when luteal regression occurs, is provided shortly after ovulation, serum progesterone concentrations increased progressively and normal 14- to 16-day luteal phases were observed in three of four animals studied.[387]

Further evidence that luteal regression is not caused directly by a reduction of gonadotropic support to the corpus luteum has been obtained from MBH-lesioned monkeys in which plasma concentrations of LH were reduced during the luteal phase by limiting the amount of GnRH delivered per pulse.[360] Reductions in plasma LH concentrations during the early luteal phase of the menstrual cycle by 50–75% did not cause immediate luteal regression. Rather,

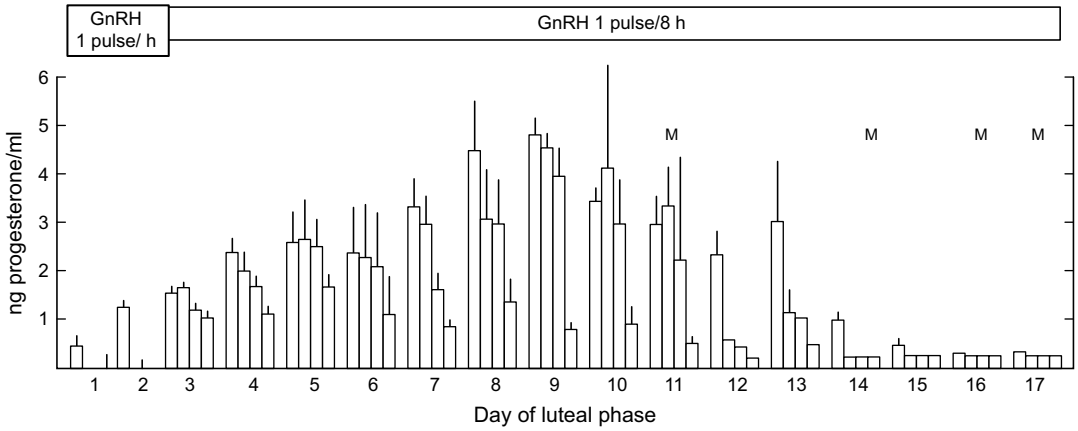

FIGURE 28.14 Response of the macaque corpus luteum to a decrease in the frequency of luteinizing hormone (LH) pulses. Medial basal hypothalamus (MBH)-lesioned rhesus monkeys received exogenous gonadotropin-releasing hormone (GnRH) at a frequency of 1 pulse/h to restore follicular development and ovulation. Shortly after ovulation, on days 2–3 of the luteal phase, the frequency of GnRH pulses was changed to one pulse every eight hours, which is typical of the mid through late luteal phase. The individual bars over each day, beginning on day 3, reflect plasma progesterone concentrations obtained 30, 60, 120, and 240 min after a GnRH pulse. Despite the reduced LH pulse frequency, normal luteal function was observed in three of four animals. *Source: Reproduced with permission from Ref. 387, Copyright 1986, The Endocrine Society.*

progesterone secretion was maintained for 6–7 days after the reductions in plasma LH concentrations. These findings suggest that during the early through mid-luteal phases of the menstrual cycle there is a surfeit of LH, whereas during the mid through late luteal phases of the menstrual cycle, an age-dependent alteration of the responsiveness of the corpus luteum to LH occurs such that a more intense gonadotropic stimulus is required to sustain luteal function. Extension of this hypothesis would predict that luteal regression at the termination of nonfertile menstrual cycles is not due to a reduction in LH secretion per se but rather to the failure of the corpus luteum to respond to ambient concentrations of LH, a conclusion that completely supports Hisaw's speculation, made 70 years ago, that "menstruation is not due necessarily to a lack or absence of pituitary gonadotropin but rather to a failure of the corpus luteum."[333]

Estradiol as a Luteolysin

Hoffman[388] noted that in humans direct injection of estradiol into the ovary containing the corpus luteum resulted in premature menstruation, whereas injection of estradiol either into the contralateral ovary or systemically did not shorten the menstrual cycle, an observation that has been confirmed by others in both humans and macaques.[389,390] These observations led to the suggestion of a "self-destruct" mechanism by which internally produced estradiol leads to the regression of the corpus luteum,[391] a hypothesis supported by the findings that concentrations of estradiol and estrone in macaque corpora lutea increase during the late luteal phase as progesterone concentrations decline.[392] The luteolytic effect of estradiol was proposed to occur directly at the level of the corpus luteum because in many instances, premature regression of the corpus luteum did not appear to be accompanied by a reduction in plasma LH concentrations.[389,393] The self-destruct hypothesis was supported further by the observation that the addition of estradiol to isolated macaque luteal cells inhibited both basal and gonadotropin-stimulated progesterone production.[394,395]

This attractive hypothesis, which would account for the observations that luteal regression does not appear to be due to a reduction in LH secretion, was questioned by subsequent observations in which sensitive bioassays were used to measure serum LH concentrations during estradiol-induced luteolysis. These studies revealed that estradiol-induced luteal regression was preceded by slight, but significant, declines in plasma LH concentrations during exposure to exogenous estradiol.[396] The physiological relevance of estradiol-induced luteal regression was also questioned by studies in MBH-lesioned monkeys, which demonstrated that exogenously administered estradiol, at dosages shown to be luteolytic in intact animals, failed to provoke premature luteal regression in monkeys in which hypophysial drive (LH support) was driven by pulsatile infusion of GnRH.[397] Unless there are totally different mechanisms responsible for luteolysis between GnRH driven and spontaneously cycling animals, a possibility that is unlikely because of the remarkable similarities of their luteal phases, it would appear that because of its failure to induce luteal regression in GnRH driven animals, estradiol is not a physiologically important luteolysin during the spontaneous luteal phase. This conclusion is supported by the findings that neither the administration of P450AROM inhibitors nor estradiol antagonists to spontaneously cycling nonhuman primates prolongs the duration of the luteal phase.[398–400]

Prostaglandins

In many subprimate species, luteal regression is caused by the production of prostaglandins by the nongravid uterus. This is clearly not the case in primates, including humans, because hysterectomy does not result in prolonged luteal phases.[401–403] However, numerous studies have shown that prostaglandins can affect luteal function. In vitro, prostaglandins E_2, I_2, and D_2 generally stimulate luteal function,[404–409] whereas prostaglandins of the F series inhibit luteal functions.[355,410–412] In vivo, infusions of prostaglandin $F_{2\alpha}$ directly into the corpus luteum have been shown to cause luteal regression.[413–416] These findings, together with the observations that luteal cells produce prostaglandins in vitro,[417–421] have led to the supposition that locally produced prostaglandins may exert direct luteolytic effects on the primate corpus luteum.

Although it is unequivocal that exogenously administered prostaglandins can induce premature luteal regression, it is uncertain whether the luteolytic effects of prostaglandins reflect physiological or pharmacological actions. Injections of indomethacin into rhesus monkeys at dosages that prevented the initiation of labor failed to prolong the life span of the corpus luteum.[422] In addition, it has been shown that direct infusion of the prostaglandin synthesis inhibitor meclofenamate into the rhesus monkey corpus luteum actually caused premature luteal regression, suggesting that prostaglandin synthesis may be required for normal luteal function as opposed to luteal regression.[423] In this regard, it has been demonstrated that direct infusion of prostaglandin E_2 into the corpus luteum inhibits prostaglandin $F_{2\alpha}$–induced luteal regression in monkeys.[424] However, infusion of prostaglandin E_2 alone during the time of luteal regression did not extend the life span of the corpus luteum, thus questioning the role of endogenous prostaglandin $F_{2\alpha}$ in the regression of the primate corpus luteum. As noted in Chapter 23, until specific antagonists of prostaglandin $F_{2\alpha}$ become available, a definitive role for this prostaglandin in the process of luteolysis in primates remains uncertain.

Cellular Correlates of Luteal Regression

Production of both estradiol and progesterone by isolated luteal cells under basal conditions and in response to tropic stimulation declines as the luteal phase progresses.[354,355] The decline in steroidogenic capacity in aging corpora lutea is accompanied by a reduction in the number of LH receptors on luteal cells, a decline in LH-sensitive adenyl cyclase activity, and a decline in LH-stimulated PKA activity.[425–428] However, it remains uncertain as to whether these changes in the transmembrane signaling system for LH are causes or consequences of luteal regression because reductions in serum progesterone concentrations may precede the decreases in LH receptors and postreceptor LH signaling. In addition to changes in LH responsiveness, luteal cells collected from corpora lutea during the late luteal phase also exhibit reduced progesterone production in response to cAMP as well as in response to the addition of pregnenolone, the immediate precursor to progesterone.[355] These data therefore suggest that the alterations in cellular function that accompany luteolysis are global in nature such that many aspects of luteal cell function are compromised. This is further supported by the results of microarray studies that the levels of hundreds of mRNAs decline during spontaneous and induced luteolysis.[367]

The histological studies of Corner describe the morphological correlates of luteal regression in macaque and human corpora lutea.[429] During the mid-luteal phase, approximately 9 days after ovulation when serum progesterone concentrations are at their zenith, the corpus luteum begins to exhibit signs of degeneration, including shrinkage of luteal cells and vacuolization of the cytoplasm. By day 11 of the luteal phase, 4–5 days before the expected time of menstruation, cell shrinkage becomes more pronounced and nuclear chromatin condensation and pyknosis are apparent. As mentioned above, the process of luteinization of granulosa cells in vivo is not completed until 5–6 days after ovulation.[307] On the basis of the morphological characteristics of luteal cells throughout the luteal phase, it would thus appear that luteolysis on a cellular level is a progressive phenomenon that may begin shortly after the process of luteinization is completed.

Stouffer and colleagues[430,431] (and Chapter 23) used flow cytometry to analyze the functional characteristics of luteal cells from rhesus monkey corpora lutea based on their sizes. At least two populations of luteal cells were distinguished, small luteal cells (15 μm diameter) and large luteal cells (>25 μm diameter). During the early luteal phase small luteal cells outnumbered large luteal cells by a ratio of 4:1, and although both small and large cells were responsive to hCG and cAMP, the absolute steroidogenic capacity of large cells exceeded that of small cells by 10- to 20-fold. As the luteal phase progressed, both the number of small and large cells declined, but the rate of decline of large cells exceeded that of small cells such that by the end of the luteal phase, the ratio of small to large cells was approximately 15:1.[431] In addition, from the mid-luteal phase onward, small cells were unresponsive to hCG and cAMP, whereas large cells retained responsiveness to both cAMP and hCG.[431] These observations indicate a gradual shift in population of steroidogenic cells as the corpus luteum ages. If the "small" luteal cells isolated by flow cytometry correspond to the shrunken granulosa–lutein cells described by Corners, it would then appear that luteal regression may be the result of a stochastic process in which individual luteal cells undergo a transformation from being gonadotropin responsive and steroidogenically competent to

gonadotropin-unresponsive cells and that at any given point in the luteal phase the functional status of the corpus luteum is determined by the relative proportions of each cell type. A progressive increase in the percentage of gonadotropin-unresponsive cells during the mid through late luteal phases of the menstrual cycle could provide a mechanism by which the corpus luteum regresses without a change in gonadotropin secretion. In this regard, the morphological changes in luteal cells that occur during the mid through late luteal phases are typical of apoptotic cell death[432] (and Chapter 23), and analyses of the human corpus luteum have revealed a progressive increase in the number of apoptotic cells during the mid through late luteal phases of the menstrual cycle.[433] The determination of whether apoptosis is the cause or the result of a loss of responsiveness to gonadotropins is not known. Neither is it known whether the diminution of gonadotropin responsiveness is due solely to a consequence of luteal cell aging, or whether it is due to the actions of other factors (such as $PGF_{2\alpha}$) that impinge on the luteal cell; this remains one of the major unanswered questions regarding luteal regression.

In summary, the formation of the corpus luteum represents the culmination of the ovarian events of the long process of gametogenesis and the beginning of the period in which the implanted embryo must be nourished and sheltered. The numerous cell divisions that granulosa cells undergo during preantral follicular development and their final differentiation into LH-responsive steroid-producing cells during the follicular phase of the menstrual cycle are not dissimilar to the morphogenesis of the elegant yellow monarch butterfly. The emergence of the corpus luteum from its follicular cocoon after ovulation begins the final episode of the ovarian cycle. Like a butterfly, the corpus luteum must fulfill a purpose that is essential for the preservation of the species, and when this function is completed, it dies. The data available to date entirely support Hisaw's conclusion, made 70 years ago, that the death of the primate corpus luteum is not due to a lack of pituitary gonadotropin secretion but rather to intrinsic changes within the corpus luteum. The simplest explanation for the regression of the primate corpus luteum is that there is an age-dependent diminution in its responsiveness to LH such that ambient concentrations of LH during the mid through late luteal phases of the menstrual cycle are no longer able to sustain its secretory activity. The final challenge continues to be the identification of the cellular mechanisms that are responsible for the age-dependent loss in the responsiveness of the corpus luteum to LH.

Neuroendocrine Control of Gonadotropin Secretion by the Corpus Luteum

During the mid-luteal phase of the human and rhesus monkey menstrual cycle, circulating progesterone is dramatically elevated and total inhibin (predominantly inhibin A) concentrations are at their highest.[47] Moreover, in the human female circulating estradiol concentrations during much of the luteal phase are approximately two-fold greater than those observed during the early follicular phase (Figure 28.1). Despite the increase in these circulating hormones of corpus luteum origin, all of which are potentially capable of inhibiting gonadotropin secretion, mean concentrations of circulating LH and FSH during the mid-luteal phase of the menstrual cycle are similar or only marginally lower than those observed at the beginning of the follicular phase.[47,434] This apparent paradox is not understood, although it has been proposed that the monkey ovary in some way is able to attenuate the profound negative feedback action of estradiol that is observed in the ovariectomized condition.[230] With the initiation of the demise in the corpus luteum late in the luteal phase, circulating levels of all three hormones decline dramatically, and the secretion of gonadotropins, in particular that of FSH, increases.

The relative importance of estradiol, progesterone, and inhibin A in relaying the ovarian negative feedback signal to pituitary gonadotropin secretion during the luteal phase of the menstrual cycle is not entirely clear. With the current data available, the least important in this regard appears to be inhibin A. Although the IV infusion of recombinant human inhibin A (1 μg/h) during the mid-luteal phase of the monkey cycle has been shown to suppress circulating FSH levels,[369] the physiological significance of this finding is difficult to evaluate because blood levels of inhibin A during treatment were not reported. Moreover, passive immunization against circulating inhibin during the luteal phase of the menstrual cycle in a closely related macaque was without effect on FSH secretion.[435] Twice daily SC injection of large doses of inhibin A at the time of the luteal–follicular transition in the rhesus monkey when FSH levels were rising resulted in selective suppression in the secretion of this gonadotropin.[191] However, the blood levels of inhibin A produced by the injections of this recombinant hormone were one to two orders of magnitude greater than basal levels, and therefore the inhibin effect must be viewed as pharmacologic.

That estradiol is a major component of the negative ovarian feedback signal during the luteal phase is suggested by the finding that, in women, continued maintenance of mid-luteal levels of this circulating steroid into the next follicular phase prevented the initiation of the rise in FSH secretion typically observed during the luteal–follicular transition.[436] This finding is consistent with another study in women, where daily administration of tamoxifen during the luteal–follicular transition resulted in a further increase in FSH secretion.[437] In the brain, tamoxifen is considered to be an ER antagonist.[438]

Although analogous approaches to address the importance of progesterone as a negative feedback regulator of gonadotropin secretion during the luteal phase has not been undertaken, a contributing role of progesterone in this regard may be inferred from our understanding of the role of this steroid in regulating LH/GnRH pulse frequency on the one hand, and the impact of changes in GnRH pulse frequency in mediating steroid feedback on gonadotropin secretion on the other. In contrast to the unremarkable changes in mean gonadotropin levels during the luteal phase of the menstrual cycle, the pulsatile mode of LH secretion is profoundly modulated at this stage of the cycle. Whereas an LH pulse frequency of approximately 1 pulse/h is observed during the greater part of the follicular phase, this may be reduced to as few as 1 pulse/8 h in the mid-luteal phase.[439–443] The deceleration of LH pulse frequency by progesterone may be readily demonstrated by experimentally imposing luteal phase concentrations of progesterone during the follicular phase of the menstrual cycle in normal women.[86] Maintenance of a low, mid-luteal phase GnRH pulse frequency during the luteal–follicular transition in GnRH-deficient women in whom the menstrual cycle is being driven by pulsatile GnRH replacement dampens the initial rise in FSH secretion normally observed at this stage of the cycle.[444]

The action of the progesterone to decelerate pulsatile GnRH release, which has been demonstrated empirically in the ewe (see Chapter 27), is unlikely to be mediated directly on GnRH neurons as there is little evidence for expression of the nuclear PR in these cells, including those of the African green monkey[445] (and Chapter 11). The finding that administration of naloxone, a generalized opioid receptor antagonist, during the luteal phase of the menstrual cycle in humans and monkeys results in an increase in LH pulse frequency,[87,446–448] and reverses the deceleration in LH pulse frequency induced by progesterone administration to women in the follicular phase,[449] provides compelling evidence for the view that signaling by endogenous opioids in the hypothalamus mediates the action of progesterone on GnRH release. In view of the emerging hypothesis that the arcuate KNDy neurons are the pivotal component of the GnRH pulse generator (see above), it is tempting to speculate that progesterone action in this regard may be directly on the GnRH pulse generator to upregulate dynorphin expression in KNDy neurons. In this regard, expression of PR in KNDy neurons has been reported for the ewe,[450] and evidence derived from studies of this species in support of progesterone action on KNDy neurons is accumulating (Chapter 27). In a study of the ovariectomized rhesus monkey, in situ expression of *KISS1* in arcuate neurons (presumably KNDy neurons) was downregulated by exposure to luteal phase blood levels of progesterone.[59] The significance of this finding is unclear, however, because (1) LH pulse frequency was not monitored, and (2) earlier studies of the monkey have shown that progesterone replacement alone to ovariectomized animals does not suppress mean levels of LH.[238] Another endorphin that has been implicated in the progesterone-induced deceleration of GnRH/LH pulse frequency in the rhesus monkey is β-endorphin, encoded by the *pro-opiomelanocortin* gene (*POMC*) that is also expressed by neurons in the arcuate nucleus (non-KNDy neurons).[451] β-endorphin levels in pituitary portal blood (and therefore perhaps also in the region of the arcuate nucleus and median eminence) of the monkey during the menstrual cycle are highest in the luteal phase.[452] β-endorphin, in contrast to dynorphin, has only low affinity for kappa opioid receptors,[85] and the mechanisms by which the putative action of these two EOPs retard GnRH pulse frequency are likely to be different. In this regard, GnRH neurons in the primate hypothalamus appear to be directly contacted by β-endorphin terminals.[453]

The negative feedback mechanisms utilized by estradiol to suppress FSH and LH secretion during the luteal phase are presumably the same as those discussed above that are operative during the follicular phase. As for the follicular phase, however, the relative importance of hypothalamic versus pituitary sites for the negative feedback action of this steroid during the luteal phase is unresolved. Nevertheless, it is clear from studies with hypophysiotropically clamped GnRH-deficient women and female monkeys that negative feedback by estradiol at the level of the pituitary is sufficient to regulate the secretion of both LH and FSH during the luteal phase of the cycle.[223–225]

Although the contribution of inhibin A to the control of gonadotropin secretion by the primate corpus luteum is unclear, any negative feedback by this ovarian peptide is likely to be exerted selectively on FSH secretion and primarily by a pituitary site of action. This view is based on studies of the hypophysiotropically clamped male rhesus monkey. Accordingly, castration in this model where the pulsatile GnRH drive to the pituitary is clamped at the male frequency of 1 pulse/3 h results in a marked monotropic rise in FSH that may be prevented by replacement with recombinant human inhibin A that maintains circulating inhibin A at levels observed for inhibin B prior to removal of the testis.[454] It should be noted, however, that circulating levels of inhibin B in the adult male monkey are approximately an order of magnitude greater than those observed for inhibin A in the luteal phase.[454,455] As established in nonprimate species, the action of inhibin to suppress FSH secretion is due to its abrogating an intrapituitary activin tone, which is stimulatory to FSH synthesis and release (see Chapter 10).

As with the mechanism underlying the initiation of the preovulatory LH surge, it appears from the foregoing considerations that there are likely to be major

redundancies in the negative feedback control system utilized by the primate corpus luteum to control gonadotropin secretion. In this regard, although the action of progesterone to robustly decelerate GnRH/LH pulse frequency is conserved across mammalian species exhibiting a prolonged luteal phase (see Chapter 33), a deceleration in GnRH/LH pulse frequency is not essential for either normal corpus luteum function or for preventing a premature FSH rise during the luteal phase of the cycle that would in turn advance the next wave of follicle differentiation. In both GnRH-deficient human and rhesus monkeys, the dynamics of menstrual cycles driven by pulsatile exogenous GnRH replacement at an unchanging frequency typical of the follicular phase (approximately 1 pulse/h) are essentially indistinguishable to those of cycles in normal subjects.[223–225] Notwithstanding, it has been suggested that a reduction of GnRH pulse frequency during the luteal phase may be necessary for optimal follicular development during the subsequent follicular phases. However, this thesis has not been investigated in great detail.

One other aspect of the neuroendocrinology of the luteal phase that needs to be mentioned is that during this phase of the cycle the hypothalamic-pituitary unit is unable to respond to the positive feedback action of estradiol.[456] That progesterone is responsible for this phenomenon was convincingly demonstrated in the monkey by the finding that estrogen-induced LH surges in the follicular phase of the cycle are blocked by prior exposure to luteal-phase levels of progesterone.[456] This action of progesterone would be brought into play in luteal phases where circulating estradiol levels exceed the strength-duration requirements for positive feedback of the steroid and may be particularly significant in the human female where luteal-phase levels of circulating estradiol are characteristically elevated. The mechanism underlying this action of progesterone remains to be established (for further details, see Chapter 45 in the previous edition).

MATERNAL RECOGNITION OF PREGNANCY

In the absence of a successful pregnancy, the primate corpus luteum has a finite 14- to 16-day life span, but its secretory activity is prolonged beyond this duration when successful conception occurs. Indeed, the abrupt increase in serum progesterone concentrations that was seen in pregnant rhesus monkeys when spontaneous luteal regression normally would have occurred led Neill et al.[457] to conclude that the corpus luteum of the menstrual cycle is "rescued" during early pregnancy.

The findings by Ascheim and Zondek in 1927[19] that urine of pregnant women contained a substance that stimulated luteinization in rats was the first evidence of a specific luteotropic factor associated with pregnancy, an observation that shortly thereafter resulted in the development of the first test for pregnancy. Because the biological activity of extracts of urine from pregnant women differed from that of extracts of urine from postmenopausal women, which also possessed the ability to stimulate follicular growth, Zondek[20] proposed the existence of two hormones, one with FSH activity and called prolan A at the time and the other with LH activity and called prolan B. Although Zondek initially proposed that both prolan A and B were of pituitary origin, the observations by Gey et al.[458] and Jones et al.[459] that cultured placenta trophoblast cells produced prolan B provided definitive evidence for the placental production and secretion of this gonadotropin, which subsequently became known as hCG.

As mentioned above, the insightful studies by Hisaw in 1944[333] demonstrated that injections of CG into rhesus monkeys during the luteal phase prolonged the functional life span of the corpus luteum, a seminal observation that was made without the benefit of chemical assays for either estradiol or progesterone. Based on careful monitoring of menstrual bleeding, coloration of sex skin, and characteristics of the vaginal mucosa, Hisaw correctly deduced that CG stimulated both estradiol and progesterone secretion by the corpus luteum. On the basis of these studies, he concluded that the newly formed corpus luteum is maintained by the pituitary gonadotropin, whereas the extension of the functional life span of the corpus luteum was a direct result of placental production of CG.

Role of CG in the Rescue of the Corpus Luteum

The requirement of CG for the prolongation of the life span of the corpus luteum was convincingly demonstrated by the findings that its neutralization by either active or passive immunization resulted in failure to support pregnancy.[460–463] Although early studies in both humans and nonhuman primates revealed a temporal association between rising concentrations of CG in peripheral plasma and progesterone and estradiol concentrations, a direct cause–effect relationship between the secretion of CG and the rescue of the corpus luteum was confounded by two observations. First, in some studies, increases in plasma concentrations of CG as indexed by elevated progesterone levels in the circulation occurred after the rescue of the corpus luteum.[464,465] Second, despite rising concentrations of CG, the heightened response of the corpus luteum was transient such that progesterone secretion declined in the presence of increasing concentrations of CG in blood.[391]

The lack of a precise relationship between the initial detection of CG in plasma and luteal rescue was most likely due to the insensitivity of early bioassays for CG. Using sensitive radioimmunoassays for rhesus CG, Hodgen et al.[466]

and Atkinson et al.[467] demonstrated that CG concentrations in plasma were detectable, on average, 10–11 days after ovulation (Figure 28.15). However, this rise occurred 1 day after the initial rise in progesterone secretion, making it difficult to establish a causal role for the appearance of CG in the blood and the rescue of the corpus luteum. Using a very sensitive immunoassay, Baird et al.[468] demonstrated that in humans hCG in urine can be detected 7–8 days after ovulation when increases in urinary excretion of estradiol and progesterone metabolites also become apparent. It is well documented that human and nonhuman primate blastocysts can produce CG in vitro.[469–471] Although it has been suggested that production of CG by preimplantation embryos and local delivery of the gonadotropin from the uterus to the ovary may contribute to the rescue of the corpus luteum,[472,473] direct injections of small amounts of hCG into the uterine cavity of rhesus monkeys failed to stimulate progesterone secretion.[474] As concluded by Ross,[475] the most likely explanation for the slight temporal discrepancy between enhanced progesterone secretion by the corpus luteum and the initial detection of CG in blood is that the corpus luteum is more sensitive to low concentrations of CG than are existing assays.

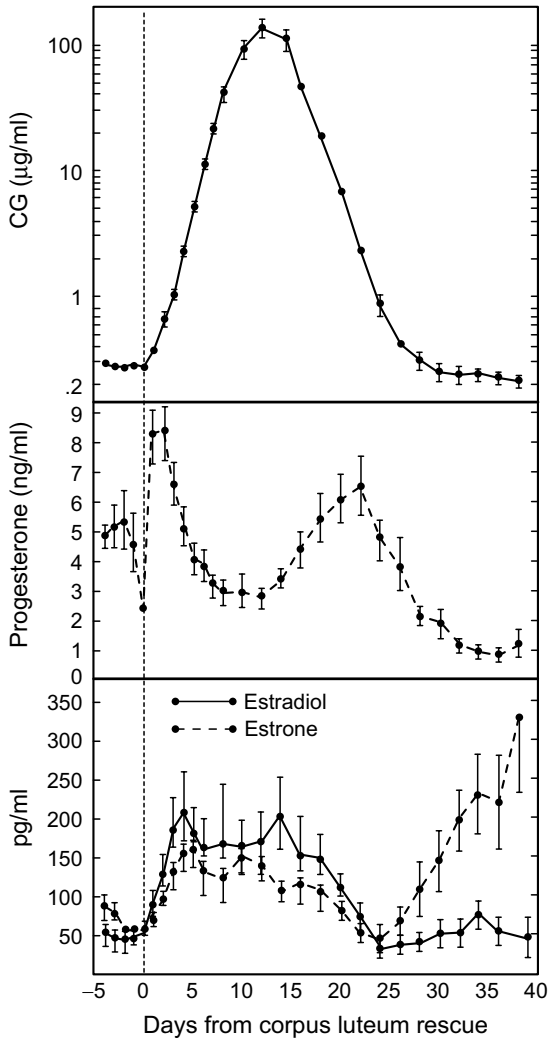

FIGURE 28.15 Patterns of serum chorionic gonadotropin (CG), progesterone, and estrogen concentrations during early pregnancy in the rhesus monkey. Results are standardized to the time of rescue of the corpus luteum (day 0) that occurs on average 10–11 days after ovulation. Note the decline in serum progesterone that occurs after the first week of pregnancy despite rising concentrations of CG. By contrast, estrogen concentrations parallel the temporal pattern of CG. *Source: Reproduced with permission from Ref. 467, Copyright 1975, The Society for the Study of Reproduction.*

More perplexing are the numerous observations that enhanced progesterone secretion by the corpus luteum is not sustained despite rising titers of CG in plasma,[476,477] a phenomenon that also occurs in response to the administration of exogenous CG.[345,478,479] Neill and Knobil[479] proposed that the decline in steroidogenesis after initial exposure to CG might be due to depletion of intracellular substrates required for steroidogenesis. More recently, it has been shown that prolonged stimulation of rhesus monkeys during the luteal phase by hCG in vivo results in a progressive decrease in the ability of hCG to stimulate adenylate cyclase activity in membranes isolated from corpora lutea.[480] This process of desensitization could account for the progressive decline in the secretion of progesterone by the corpus luteum in response to the progressive increases in plasma concentrations of CG that occur during early pregnancy. However, as deduced by Hisaw[333] and subsequently demonstrated later by others,[481,482] the progressive decline in progesterone production in response to hCG is not accompanied by a parallel decline in estradiol secretion. These observations indicate that individual CG-dependent biosynthetic pathways of the corpus luteum may be differentially affected by luteal cell aging, a hypothesis that is supported by the observation of an age-dependent attenuation of the responsiveness of selected mRNAs to CG stimulation[481] as well as differences in the temporal pattern of expression of mRNAs during hCG administration.[482] These findings indicate that luteal cell aging is associated with a selective loss of individual gonadotropin-sensitive biosynthetic pathways and agree well with the physiological data that luteal rescue (and exogenous hCG treatment) is associated with continuous stimulation of estradiol secretion but only transient stimulation of progesterone secretion and that the corpus luteum becomes less responsive to exogenous CG as the luteal phase progresses.[345,478] Diminished progesterone production by the corpus luteum therefore does not appear to be due to an absolute refractoriness to CG but rather to a selective loss in specific luteal cell functions.

Mechanism of Action of CG

CG and LH share identical α subunits, whereas their β subunits differ in size and pattern of glycosylation.[483]

The additional 30-amino acid sequence at the carboxyl terminal of CG-β together with the different glycosylation pattern results in a plasma half-life of CG that is substantially longer (36 h versus approximately 20 min) than that of LH.[484–486] Both CG and LH appear to occupy the same receptor[487] on luteal cells, both stimulate adenylate cyclase activity in luteal cell membranes,[353] and both stimulate progesterone secretion by isolated luteal cells.[488] The paradox regarding the rescue of the corpus luteum therefore is why the corpus luteum regresses in the presence of LH yet the similar molecule CG is able to prolong its functional life span.

Three hypotheses have been developed to account for the differential responses of the primate corpus luteum to LH and CG. One hypothesis is that the patterns of the secretory dynamics of LH and CG provide different gonadotropic stimuli to the corpus luteum. This hypothesis relates to the fact that whereas plasma concentrations of LH intermittently fall to very low values during the luteal phase due to the pulsatile nature of LH secretion by the pituitary gland, plasma concentrations of CG do not fall to these low levels due to both the continuous secretion of CG by the placenta and its longer circulatory half-life.[489] It is thus possible that continuous exposure of the corpus luteum to CG provides a more intense gonadotropic stimulus to the corpus luteum than does the episodic stimuli provided by LH. A second hypothesis is that although CG and LH are structurally similar, the amino acid differences in the β chains of CG and LH as well as differences in the extent of glycosylation and sulfation[490] could result in an inherent difference in biological activity between the two hormones. This hypothesis is based largely on studies of rodent and ovine systems that demonstrated that the time course of steroid production in response to hCG was significantly prolonged when compared with that of ovine LH, suggesting that the dynamics of the interaction of CG with the CG/LH receptor and/or the subsequent activation of intracellular signaling pathways may differ from that of LH.[491,492] More recent studies using recombinant hCG and LH in a variety of cell-based bioassays have indicated that although there is overlap in the activities of hCG and LH, on an equimolar basis, hCG was more effective in the stimulation of cAMP production while LH was more effective in the activation of ERK and AKT signaling pathways.[493] A third hypothesis is that the aging corpus luteum exhibits a diminished responsiveness to gonadotropins such that the ambient concentrations of LH present during the mid through late luteal phases of the menstrual cycle are unable to sustain its functions[360] and that the functional life span of the corpus luteum is prolonged in early pregnancy because the exponentially increasing plasma concentrations of CG transiently overcome the diminishing responsiveness of the aging corpus luteum to LH.

These hypotheses were tested in cynomolgus monkeys. In this study, monkeys received intravenous infusions of either LH or CG in a continuous or an exponentially increasing manner beginning during the mid-luteal phase of the menstrual cycle and continuing beyond the expected time of luteal regression.[494] As shown in the left panels of Figure 28.16, a continuous infusion of either LH (top) or CG (bottom), which resulted in bioactive LH/CG concentrations 10 times greater than endogenous LH concentrations, did not prolong the functional life span of the corpus luteum. These findings reinforce the notion that luteal regression at the end of nonfertile menstrual cycles is the result of a decrease in the responsiveness of the aging corpus luteum to LH because superimposition of exogenous LH or CG at ten-fold greater concentrations than endogenous LH concentrations did not prolong the functional activity of the corpus luteum. Also, these results do not support the hypothesis that a simple switch from pulsatile LH secretion to the continuous secretion of CG is sufficient to rescue the corpus luteum because timely luteal regression occurred in the animals that received a continuous (i.e., nonpulsatile) infusion of LH.

In contrast, exponentially increasing concentrations of LH or CG (Figure 28.16, right panels) prolonged progesterone production beyond the expected time of luteal regression. However, the responses to LH and CG were not identical because although the pattern of progesterone production in animals that received the exponentially increasing regimen of CG was similar to that of pregnant animals, the cumulative progesterone levels in response to the exponentially increasing LH infusion were 40% lower than those seen in the animals that received hCG, despite the fact that bioactive plasma concentrations of both gonadotropins were comparable throughout the duration of the infusions. Thus, it appears that luteal rescue requires CG rather than LH and that the corpus luteum be exposed to an exponential increase of this gonadotropin. The reason CG was more effective than LH in this experimental paradigm remains to be answered, although as noted above, on an equimolar basis hCG appears to be more effective than LH in stimulating cAMP production in cell-based bioassays.[493] Thus, hCG would be expected to produce a greater cAMP response and result in greater progesterone production in response to hCG when compared with that of LH, consistent with the findings shown in Figure 28.16.

Interestingly, the duration of CG production by the placenta differs among primate species.[495] In humans, hCG concentrations reach a maximum during the first trimester of pregnancy, decline thereafter, but remain detectable throughout the entire duration of pregnancy. A similar pattern of CG production is seen in apes, with the exception that the absolute plasma concentrations are an order of magnitude less than those seen in humans.

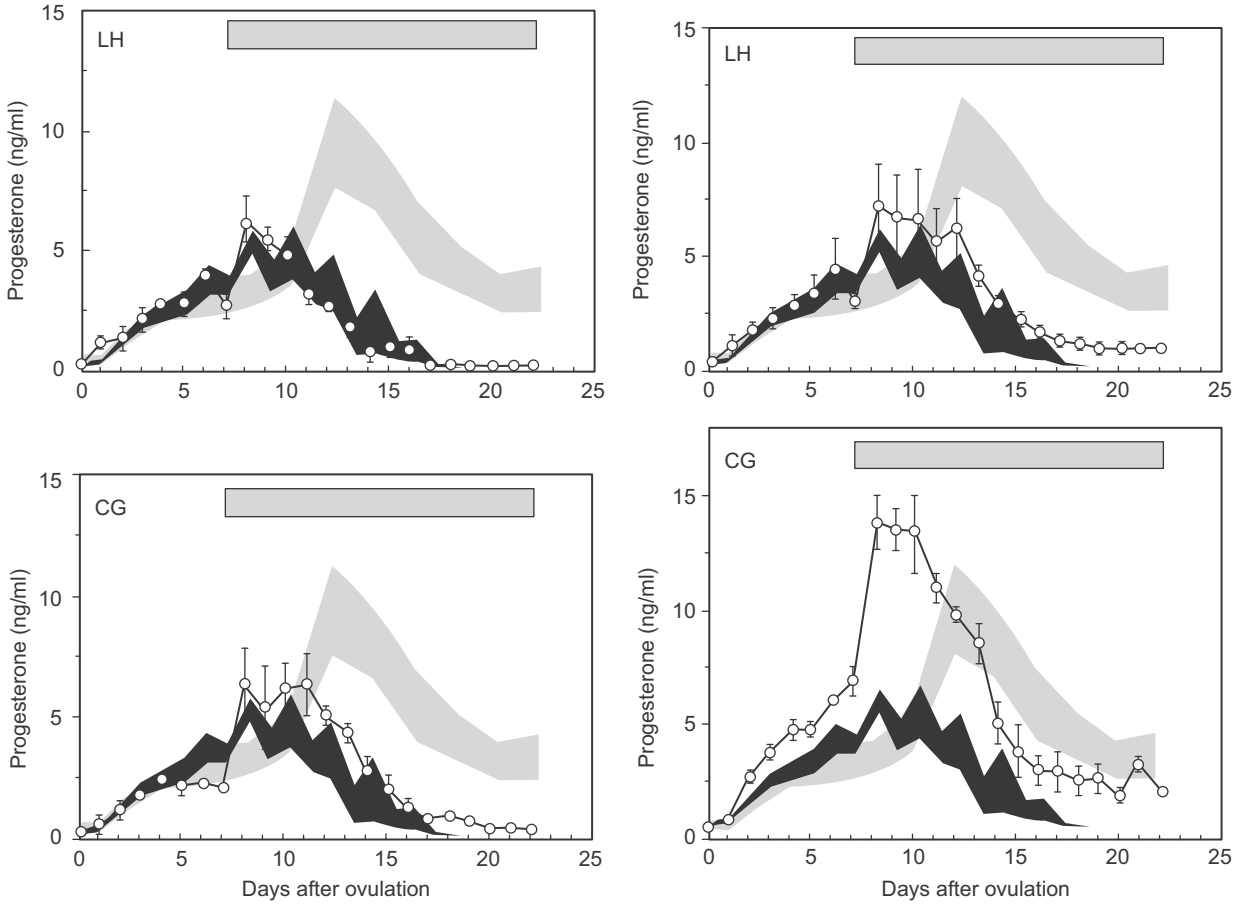

FIGURE 28.16 Responses of the primate corpus luteum to exogenous luteinizing hormone (LH) and human chorionic gonadotropin (hCG). The duration of each gonadotropin treatment is shown by the shaded rectangle at the top of each panel. The left panels illustrate serum progesterone in animals that received intravenous infusions of LH and hCG at a constant rate. The right panels illustrate serum progesterone in animals that received intravenous infusions of LH and hCG at an exponentially increasing rate. The dark shaded area encompasses the means +/− 1 standard error of the mean (SEM) of serum progesterone concentrations during luteal phases of three control monkeys, and the light shaded area encompasses the means +/− 1 SEM of serum progesterone concentrations during luteal phases of three pregnant monkeys. *Source: Redrawn with permission from Ref. 494, Copyright 1999, National Academy of Sciences USA.*

In baboons and macaques, CG concentrations decline to near undetectable levels during mid-pregnancy. Whether there are functional requirements that underlie the different patterns of CG secretion, such as direct actions on the conceptus or uterus,[496,497] are not known.

The Luteal–Placental Shift

The sole function of the corpus luteum in the maintenance of pregnancy is the production of steroid hormones, because women with ovarian failure and ovariectomized monkeys can achieve successful term pregnancies after embryo transfer provided they receive exogenous estradiol and progesterone.[498,499] In both humans and nonhuman primates, the corpus luteum is essential until the placenta assumes the major role of steroid secretion. Removal of the human corpus luteum before the fifth week after implantation resulted in a decline in serum progesterone concentrations and abortion.[296] In rhesus monkeys it has been shown that successful pregnancies can be observed after the removal of the corpus luteum as early as 1 week after implantation despite a dramatic fall in serum progesterone and estradiol concentrations.[500] That abortions can be prevented by replacement with progesterone alone in humans and nonhuman primates after luteectomy indicates that this steroid, so aptly named by Corner and Allen for its progestational effects, is the sole luteal substance required for the maintenance of early pregnancy.[501]

The fate of the corpus luteum during pregnancy has been followed in humans by the measurement of 17α-hydroxyprogesterone in blood. Because the 17α-hydroxylase enzyme is present in the corpus luteum but not in the placenta, presumably all 17α-hydroxyprogesterone in blood is derived from the corpus luteum. Plasma concentrations of 17α-hydroxyprogesterone peak during the fourth and fifth week of gestation and decline thereafter, whereas

serum progesterone concentrations continue to rise after the fifth week of gestation, indicating that from week 5 onward, the major source of circulating progesterone is the placenta.[477] Ovariectomy of rhesus monkeys on day 25 of pregnancy does not result in a dramatic fall in serum progesterone concentrations, indicating that by this time the placenta is the primary source of progesterone in this species.[391]

Although steroid secretion by the corpus luteum wanes during early pregnancy, it does not appear that the corpus luteum becomes totally nonfunctional. It has been shown in humans at term pregnancy that venous blood draining the ovary bearing the corpus luteum contains greater concentrations of both progesterone and relaxin than that found in blood from the contralateral ovary and the peripheral circulation.[318] In postpartum monkeys, progesterone concentrations in ovarian venous blood from the ovary that contains the corpus luteum are greater than those found in blood from either the contralateral ovary or the peripheral circulation.[362] The fact that lactating animals have higher concentrations of progesterone in the peripheral circulation as well as in the blood draining the corpus luteum–bearing ovary suggests that prolactin may contribute to the maintenance of the low activity of luteal function observed during pregnancy and immediately after parturition. The physiological significance of the persistence of luteal activity throughout pregnancy and after parturition is not known in view of the observations that successful pregnancies occur when the corpus luteum is removed after the luteal–placental shift.

CONCLUSION

As noted in our introductory comments, the menstrual cycle, and hence the preservation of our species, is dependent upon the physiological signaling processes that occur between the ovaries, pituitary, and brain. The result is a remarkable orchestration of perfectly timed events that culminate in the production and ovulation of a mature oocyte, the formation of the corpus luteum with the concomitant preparation of the uterine endometrium for the implantation of a fertilized oocyte, and the establishment and maintenance of pregnancy. As we have discussed in this chapter, classical negative and positive feedback loops between ovarian hormones, primarily estradiol, and the hypothalamic-pituitary unit coupled with development-dependent changes in gonadotropin responsiveness of the ovary to FSH and LH are sufficient to account for the initiation of preovulatory follicular development, the selection of a preovulatory follicle, ovulation, and the formation and regression of the corpus luteum. The extension of the corpus luteum's functional lifespan during early pregnancy is mediated by the secretion of hCG by the placenta, which overcomes the diminishing responsiveness of the aging corpus luteum to LH. This knowledge has directly led to the development of novel ovulation induction regimens for women that minimize the production of multiple oocytes and the morbidities associated with multiple births. As our knowledge continues to increase in the areas of preantral folliculogenesis and autocrine and paracrine regulation of follicular development (see below), additional therapies for ovarian dysfunction (primary ovarian insufficiency, polycystic ovary syndrome, etc.) are likely to emerge in the future.

With respect to important unanswered questions, at the ovarian level we are just beginning to understand the mechanisms responsible for activation of primordial follicles and the control of preantral folliculogenesis and atresia. The recent findings that the PI3-K signaling pathway plays a major role in primordial follicle activation now provides a target to explore this essential component of follicular growth and reproductive lifespan. What remains to be answered is the identification of the ligand(s) that regulate the PI3-K pathway in primordial follicles and how their production is regulated to control the timing of primordial follicle activation (Chapter 21).

Likewise, the identification of local autocrine and/or paracrine factors that control the growth of both gonadotropin-independent preantral folliculogenesis as well as gonadotropin-dependent preovulatory folliculogenesis could lead to novel strategies in controlling anovulatory folliculogenesis. In this regard, in vitro tissue culture studies with isolated ovarian granulosa and theca cells have led to the identification of a number of autocrine and paracrine agents (IGFs, activins, inhibins, steroids, etc.) that modulate proliferation, differentiation, and apoptosis. However, these tissue culture studies are typically conducted in serum-free culture medium because the presence of serum can adversely affect cellular responses.[502,503] Given that the ovary is likely exposed to serum proteins in vivo, it will be essential to develop novel in vivo approaches to document the physiological actions of these locally acting factors. Although gene targeting approaches have provided in vivo models to explore the effects of autocrine and paracrine factors (see Chapter 21), it will ultimately be necessary to translate information gained from murine models to higher primates.

Another major unanswered question in primate ovarian physiology is the elucidation of mechanism(s) that are responsible for the regulation of luteal regression at the termination of nonfertile menstrual cycles. Is this due simply to an age-related diminution of LH responsiveness (as we believe), or is it the result of the actions of luteolytic agents (prostaglandins, steroids) that impair LH signaling?

Folliculogenesis, ovulation, and luteal function are absolutely dependent upon the pituitary receiving an intermittent stimulatory GnRH signal from the brain that is produced by the hypothalamic GnRH pulse

generator. In fact, an unchanging pulsatile GnRH input to the primate pituitary is sufficient to allow ovarian cyclicity and ovulation to unfold: an observation that led Knobil in 1980 to propose that the role of this releasing factor in the control of the menstrual cycle was permissive. In the absence of a pulsatile GnRH drive to the pituitary, menstrual cyclicity ceases and a state of anovulation, hypoestrogenemia, and amenorrhea ensues. Although the importance of hypothalamic GnRH pulse generation has been appreciated for decades, a promising model to account for the neurobiology underlying this mode of GnRH release has only recently begun to emerge. This model, developed primarily from studies of nonprimates, posits that the KNDy neurons in the arcuate nucleus of the MBH are the cornerstone of the GnRH pulse generator, and that the output of this signal generator is imposed upon a network of GnRH neurons by kisspeptin. Because of the fundamental role of pulsatile GnRH release to all phases of the menstrual cycle, the major question in the context of the neural control of the adult ovary relates first to the validity of this model and the extent to which it can be translated to the human female. If the answer to this question is that the model is compelling and translatable to primates, then it will be important to elucidate the specific neurobiological mechanisms whereby neurokinin B and dynorphin (and perhaps other neuropeptides expressed by KNDy neurons) generate coordinated pulsatility within the network of arcuate KNDy neurons, and how the selective kisspeptin output of this network to the GnRH network is achieved. It will also be important to determine the role, if any, of non-KNDy neurons and other hypothalamic elements such as glia in the context of pulse generation. In our view, the importance of understanding the machinery of the hypothalamic GnRH pulse generator dwarfs all other questions relating to the neural control of the menstrual cycle. This is because (1) GnRH pulse generation is the only hypothalamic input to the pituitary that is obligatory for menstrual cyclicity, (2) the capacity of the human hypothalamus to generate a preovulatory GnRH surge is unclear, and (3) while the relative importance of hypothalamic versus pituitary sites of estradiol feedback on gonadotropin secretion are intellectually interesting, they are unlikely to be answered in an in vivo setting with contemporary technology. Understanding the neurobiology of GnRH pulse generation and the hypothalamic signals that regulate pulse amplitude and pulse frequency may offer new therapeutic approaches to disorders of the menstrual cycle that are occasioned by inappropriate GnRH pulse generation such as PCOS.

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CHAPTER

29

Pathophysiology of Ovarian Function in the Human Female

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INTRODUCTION

Female infertility has been described and its causes speculated upon for centuries. Indeed, there are several references to infertility in the bible, the earliest being in Genesis 16: "Now Sarai Abram's wife bare him no children". Hippocrates (c. 460–c. 375 BC) refers to infertility in a series of essays entitled "The influence of climate, water supply and situation on health". This is almost certainly one of the first recorded references to the effect of environment (nutrition) on reproduction. Commenting on the influence of obesity on fertility amongst the Scythians, he notes, "The girls get amazingly flabby and podgy… people of such constitution cannot be prolific… fatness and flabbiness are to blame. The womb is unable to receive the semen and they menstruate infrequently and little".[1]

A concise and lucid history of the discovery of the mammalian ovary is provided by Roger Short,[2,3] and further insight into the key landmarks in understanding the physiology of the ovary can be found in Chapter 28. Short includes a reference to the notion that it was Aristotle who was the first to describe the ovaries in an article describing the spaying of sows.[4] However, it was not until the seventeenth century that the seminal work of Regnier de Graaf provided the basis for our current understanding of the functional anatomy of the ovary.[5] The term "Graafian follicle" is still used to describe the preovulatory follicle even though de Graaf, at the time, considered that the structure of what we now know as the mature follicle (comprising the somatic cells and oocyte) was the egg itself. The distinguished anatomist Malpighi, later in that century, observed that the follicular structure that de Graaf had referred to as the egg retained the ovary despite evidence of ovulation. It was Prévost and Dumas in 1824 who concluded that the follicle contained the oocyte but was not the egg itself.[3]

Recognition of the hormonal regulation of ovarian function had to wait until the twentieth century. Crowe and colleagues (1910), followed by P.E. Smith (1927),[6] demonstrated that hypophysectomy in the rat resulted in regression of the gonads (reviewed by Greep[7]). Smith (1930) also showed that pituitary transplant in hypophysectomized animals restored gonadal function. Zondek and Ascheim (1927)[8] provided evidence for gonadotropic substances in the pituitary and suggested that there were two distinct gonadotropins,[9,10] a notion that was later confirmed in the classic studies of Fevold and colleagues.[11] Purification of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary extracts and from urine followed much later.[12–14]

Regarding the assay of reproductive hormones, Levin and Tyndale (1936) described a method for measuring gonadotropins in urine.[15] Applying this methodology, Werner (1941)[15] reported cyclical variation in gonadotropin excretion (as well as cyclical changes in sex hormone excretion) in five women with regular menses, who were studied over a 3- to 4-month period. This method was extremely laborious, involving collection of urine over 48 h periods, an acidification step, followed by extraction of protein, partial purification, and then an in vivo mouse bioassay to examine gonadotropic action.[15] Further assays for gonadotropins in urine were developed by Steelman and Pohley (1953)[16] and Brown (1955).[17]

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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Gonadotropin measurements in urine later revealed abnormalities in women with disorders of ovulation. It was Ingersoll and McArthur (1959)[18] who first noted excessive excretion of LH in the urine of women with polycystic ovary syndrome (PCOS). In this study and over much of the following decade, the measurement of gonadotropin activity in the extracted protein fraction of urine still relied on the use of a subsequent in vivo bioassay.[19]

The advent of the radioimmunoassay for gonadotropins and sex steroids in the 1960s and its subsequent widespread application in the early 1970s were key factors in advancing our understanding of disorders of ovulation in women as well as shedding new light on the physiology of the hypothalamic–pituitary–ovarian (HPO) axis. The ability to measure hormones in small volumes of blood allowed researchers to distinguish easily between a primary ovarian cause (low estradiol and high FSH) and hypothalamic–pituitary causes of anovulation (low estradiol and low or normal gonadotropins). The demonstration of the pulsatile nature of gonadotropin secretion and the ability to show, convincingly, that disorders of ovulation associated with gonadotropin deficiency were often hypothalamic in origin (i.e., an aberrant pulsatile pattern, indicating disordered gonadotropin-releasing hormone (GnRH) secretion) represent a further, significant advance in understanding the pathophysiology of the HPO axis. Knobil and colleagues, in a study of LH secretion in the ovariectomized rhesus monkey,[20] proposed that LH pulsatility was dependent on an LH-releasing factor produced by the brain. In the same year, Dolais and coworkers reported LH pulses in women.[21] These studies were followed by those of Midgley and Jaffe[22,23] and Wide,[24] who were amongst the pioneers who produced meticulous studies to define the episodic nature of LH and FSH secretion by using highly sensitive immunoassays, and it was then possible to link these findings to the discovery of the identity of GnRH in 1971 (further discussed in this chapter).

At about the same time as the elucidation of the structure of GnRH, the identity of prolactin as a hormone that had a distinctly different structure from growth hormone was reported.[25] A bioassay for prolactin using the pigeon crop sac had been developed as early as 1933 by Riddle and colleagues,[26] and a bioassay for lactogenic hormones (in which there was inevitably cross-reactivity with growth hormone and human placental lactogen) was used for many years.[27] Subsequent development and application of a specific radioimmunoassay for prolactin[28] led to the realization that hyperprolactinemia was a very common cause of amenorrhea in women.[29] MacLeod (1970)[30] had shown that the likely hypothalamic factor that kept prolactin under tonic inhibitory control was dopamine. Coincidentally, the long-acting dopamine agonist bromocriptine became available for the management of Parkinson syndrome, and it was shown that, in small doses, bromocriptine effectively reduced prolactin levels and restored ovulation in women with hyperprolactinemic amenorrhea.[31,32]

Following the identification of the structure of GnRH by Schally and Guillemin,[33,34] it was the seminal work of Knobil and colleagues[35,36] in the 1970s that showed the importance of pulsatile administration of GnRH in restoring ovulatory menstrual cycles in primates. This led to the application of GnRH treatment to restoring ovulation and fertility to women with clinical disorders of gonadotropin secretion.[37–40] The discovery and subsequent synthesis of GnRH were important not only because of its implications for the treatment of infertility but also because it provided unique insight into the physiology of the HPO axis. For example, examination of the endocrinology of GnRH-induced ovulatory cycles in the nonhuman primate and in women with GnRH deficiency revealed not only normal cyclical patterns of estradiol and progesterone secretion but also modulations in the levels of LH and FSH despite using a fixed dose of GnRH. This demonstrated that both negative and positive steroid feedback was operating at the pituitary level and that this was sufficient to produce a normal ovulatory cycle.

Even before the advent of GnRH for therapeutic use, treatment of ovulatory disorders in women had been revolutionized in the 1960s by the use of the antiestrogen clomiphene[41–43] and, in women with gonadotropin deficiency, of exogenous gonadotropins.[44–46] These gonadotropins either were of pituitary origin or were extracted and purified from urine of pregnant (human chorionic gonadotropin–LH activity) or postmenopausal women (predominantly FSH activity).

The enormous progress, in the modern era, in our understanding of the pathophysiology of ovulatory disorders in women and their management is the subject of this chapter.

PHYSIOLOGY OF HUMAN OVARIAN FUNCTION

The normal reproductive lifespan of a woman ranges from her early teens until her early 40s, although fertility is progressively reduced in her 30s. Fertility depends on a number of key factors, of which, of course, regular ovulation is a fundamental requirement but oocyte "quality" is also a major consideration. In this chapter, the main focus will be on disorders of ovulation, which are a highly prevalent cause of infertility and may also impact other aspects of women's health. To set the scene, however, we will discuss some of the more important aspects of the physiology of the human ovary, which is intended to complement the detailed exposition of the menstrual

cycle in higher primates in Chapter 28. The principal causes of disordered ovulation will be described, and their clinical manifestations and management discussed. In an era of fertility treatment that is dominated by the use of assisted conception techniques, it is important to highlight the fact that correct identification of the cause of ovulatory disorders will, in most cases, lead to the successful use of specific treatments that target restoration of normal, mono-ovulatory menstrual cycles. This is an exciting era in reproductive physiology and endocrinology in which the uncovering of important genetic influences upon—and, in some cases, determinants of—many common reproductive endocrine disorders such as hypothalamic amenorrhea, primary ovarian insufficiency (POI), and PCOS have provided real hope of more precise methods of diagnosis and treatment.

Follicle Development in the Human Ovary

The formation of primordial (resting) follicles in the human female takes place during the last trimester of pregnancy and is essentially complete by the time of birth. Follicle formation in the primate ovary is discussed in detail in Chapters 21 and 28. In the human fetal ovary, about two million primordial follicles, from an initial pool of around five million oocytes, are present at birth and, by the time of puberty, that number has declined to an estimated 100,000–200,000.[47] Thereafter, there is a steady decline in the population of follicles throughout childhood and reproductive life as a result either of progression of follicles through the various stages of development or of loss by atresia (follicle death) (Figure 29.1).[48–51] It is evident that growing follicles also undergo atresia because the vast majority of follicles that start to grow never reach the mature preovulatory stages. The most obvious loss of follicles is seen at the antral stages, but there is also evidence that atresia may occur at any stage during development in the primate ovary. The progression of the follicle and its enclosed oocyte from the primordial stage to a mature preovulatory follicle takes several months in the human ovary[52,53] (Figure 29.2).

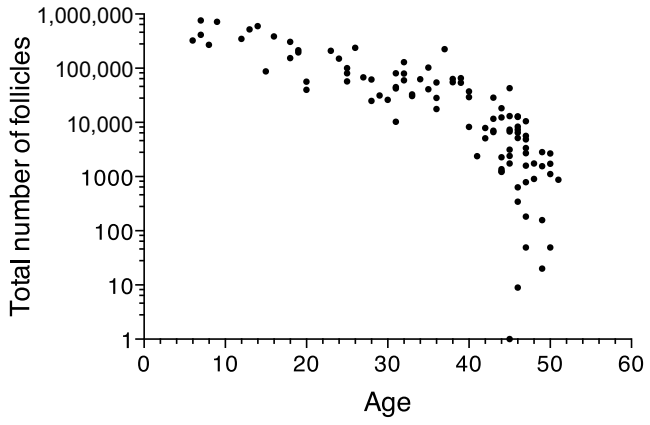

FIGURE 29.1 The decline in follicle number in the human ovary with age. *Source: Data compiled from various sources.*[48–51]

Although there are recent data to suggest that mitotically active oogonial germ cells are present in the human ovary that have the potential to generate new oocytes,[54] it is unlikely that such cells can significantly affect reproductive lifespan because the net loss of follicles clearly exceeds renewal from any stem cell pool. Nevertheless, this is an important observation and, if validated, does open the prospect of being able to "harvest" such cells for therapeutic use in restoring ovarian function in those with, or at high risk of, POI. Essentially the steady decline in follicles with age continues unabated and the rate of loss determines the age of menopause. There is an accompanying reduction, with age, in the competence of oocytes to develop into healthy embryos, as is attested by the steep rise in the rate of aneuploidy and miscarriage observed as women reach their late 30s and beyond (see Ref. 55 and Chapter 1). Nevertheless, there is enormous variation between women in the decline of the oocyte population, which is reflected in the wide range in age for the onset of menopause (40–55 years). Measurement of serum concentrations of anti-Müllerian hormone (AMH), a secretory product of granulosa cells, predominantly from large preantral and antral follicles, has gained favor in the assessment of ovarian follicle reserve, particularly in the context of predicting responses to exogenous gonadotropin stimulation in assisted reproductive technologies. However, whilst serum AMH levels broadly correlate with fertility, there is a huge variation within the normal range,[56] which (coupled with ongoing problems with standardization of the immunoassays[57]) renders such assessment unreliable at present.

Preantral Follicle Development

Preantral follicle development can progress in the absence of gonadotropin stimulation as far as the multilayered, preantral stage. Antrum formation and further progression to large antral follicles are, however, dependent on gonadotropins, and FSH in particular. Nevertheless, granulosa cells of preantral follicles do have receptors for FSH, and it can be shown that even in the very earliest stages of follicle development FSH can act as a survival factor in human ovarian tissue[58] and promote activation of primordial follicles,[59] at least in vitro. These findings suggest that FSH may play a physiological (although nonessential) role even in preantral follicle growth. It is therefore appropriate to use the terms "gonadotropin sensitive" for preantral development and "gonadotropin dependent" for the antral stages (Figure 29.2).

The rate at which primordial follicles enter the growing pool must be tightly regulated in order to ensure a steady supply of growing follicles throughout

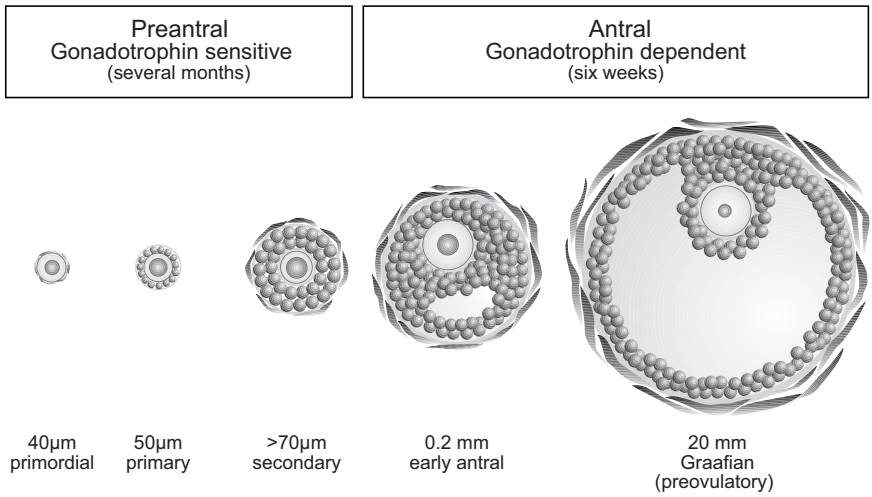

FIGURE 29.2 Stages of human follicle development.

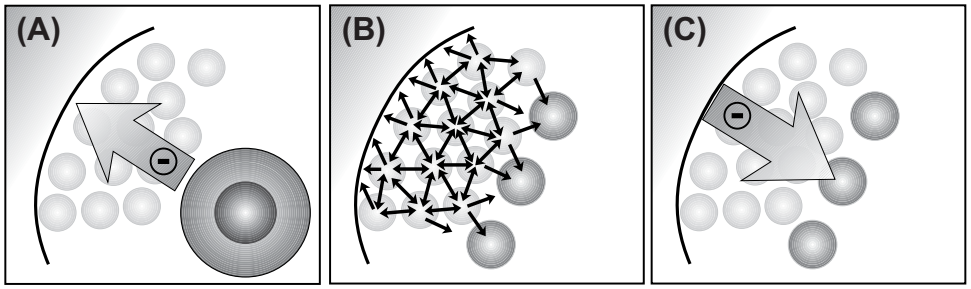

FIGURE 29.3 Possible mechanisms to provide inhibition of activation of primordial follicles in the mammalian ovary. (A) Inhibitory signal from growing follicles; (B) primordial follicles produce a local factor (or factors) that inhibits their neighbors from activating; (C) an inhibitory factor produced by the ovarian surface epithelium.[60]

reproductive life. One approach to understanding initiation of follicle development is to use mathematical modeling to test hypotheses about the mechanisms involved. Observing that primordial follicles tend to exist in clusters near the ovarian surface (whereas growing follicles were less likely to be adjacent to other follicles and more central within the ovary), Da Silva-Buttkus et al.[60] proposed that one of three possible mechanisms determined whether primordial follicles remained in the resting pool or were activated to grow (Figure 29.3). These hypotheses were that (1) primordial follicles produced an inhibitory factor so that adjacent follicles in the cluster inhibit each other, (2) growing follicles produce a factor that inhibits activation of primordial follicles, and (3) an inhibitory factor (or factors) was produced by the ovarian surface epithelium. They mapped positional coordinates of all follicles in sections of juvenile mouse ovary and computed the Euclidian distance between each and every follicle in that section. As illustrated in Figure 29.4, there was clear evidence to support the hypothesis that primordial follicles inhibit each other's growth. The more primordial neighbors in the immediate vicinity of the "target" follicle, the less the chance of it being a growing follicle; conversely, the fewer the primordial follicles in the immediate vicinity, the more likely it was to be

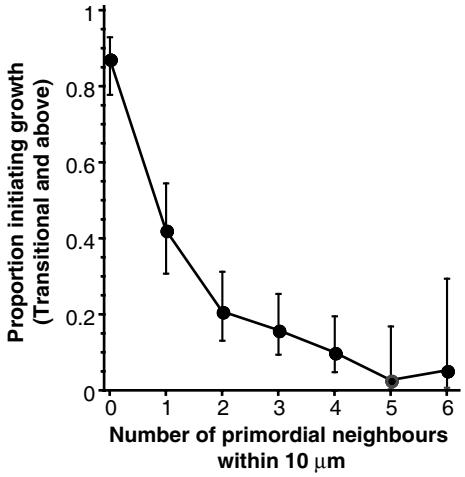

FIGURE 29.4 Neighboring primordial follicles inhibit activation of follicle growth. The proportion of follicles that are growing is inversely related to the number of primordial follicles within a 10 μm radius, suggesting that neighboring primordial follicles inhibit each other from initiating growth (juvenile mouse ovary day P8).[60]

a growing follicle. It is evident that much further work needs to be done before the key factors involved in the putative inhibitory "brake" on follicle activation can be elucidated, but among plausible candidates are growth

factors in the transforming growth factor beta (TGFβ) superfamily and/or their naturally occurring antagonists. Recent data from studies in the mouse have lent support for such a role for these paracrine factors (see the section Polycystic Ovary Syndrome and Chapter 21).

Although most of the studies of initiation of follicle development have taken place in experimental animals, results from the little work that has been done in human ovary suggest that similar phenomena prevail in the activation and growth of human preantral follicles.[52,61–63] What is clear is that the morphological changes that characterize initiation of follicle growth in rodents, domestic animals, and nonhuman primates can also be observed in the human ovary,[52,63] although there is still much to be learned about the growth factors that are implicated in early follicle development in the human ovary.[61] Importantly, it is from such studies in human ovaries that it has been possible to define aberrations in early follicle development in PCOS,[64,65] as discussed at length in the section Polycystic Ovary Syndrome.

Gonadotropin-Dependent Follicle Development

Our understanding of the later, antral stages of follicle development is much greater than that of the factors affecting early follicle growth. The physiology of antral follicle growth and the changes associated with the primate menstrual cycle are described in considerable detail in Chapter 28 and will not be reiterated here except to illustrate, briefly, the particular features of dynamic changes in the HPO axis that characterize the human ovulatory cycle (discussed further in this chapter). What is clear is that development of antral follicles to a size of between 1 and 5 mm in diameter can take place in the absence of cyclical changes in gonadotropins; however, the emergence, maturation, and ovulation of a dominant follicle require the characteristic cadence of gonadotropin secretion that is observed during the menstrual cycle. Such cyclical changes depend, in turn, on a normal pattern of secretion of GnRH, which is itself influenced by afferent inputs in the hypothalamus that, in turn, are sensitive to parameters of the external environment, particularly those related to nutritional status. It is beyond the scope of this chapter to describe the network of neural inputs to the GnRH pulse generator in detail, but suffice it to say that disorders of GnRH secretion are well-recognized causes of anovulation in women and there is increasing evidence that disturbances in afferent neural input to the GnRH neural network play a role in these clinical disorders. These will be discussed in the *Disorders of Ovulation*.

Steroidogenesis in the Human Ovary

Control of steroid hormone production during the follicular and luteal phases of the primate menstrual cycles is described extensively in Chapter 28. That discussion includes the role of insulin-like growth factors (IGFs), as physiological modulators of gonadotropin action, in ovarian steroidogenesis. Later in this chapter, we shall focus on both the possible physiological and the likely pathological implications of the actions of IGFs (and insulin itself) in the context of aberrant steroidogenesis by both theca and granulosa in PCOS.

The Human Menstrual Cycle

The cyclical changes in gonadotropins and ovarian steroids in the human menstrual cycle are summarized in Figure 29.5. Here it can clearly be seen that toward the end of the previous cycle, the steep fall in the serum levels of estradiol and progesterone, which signal the demise of the corpus luteum, is accompanied by a rise in both FSH and LH. The increase in FSH is more marked than that of LH, and it is this intercycle rise in FSH that is critical for the further development of the small cluster of antral follicles (1–5 mm in diameter) that are present at that time. In essence, in the normal early follicular phase of the cycle, FSH concentrations exceed a critical level or "threshold" for further development of that cluster of antral follicles,[67,68] and it is the follicle that is most sensitive to FSH (usually, but not invariably, the largest of the cohort) that becomes dominant and goes on toward ovulation. As estradiol levels rise steadily in the early follicular phase, a negative feedback inhibition of FSH is activated and, whereas the dominant follicle continues to develop in the face of a reduced FSH signal, the subsidiary follicles in the cohort are deprived of sufficient FSH stimulation and become atretic. This is considered to be the major mechanism underlying mono-ovulation in primates. The contribution of inhibins to the physiology of negative feedback regulation of FSH remains a somewhat controversial area. Inhibin B is mainly produced by small antral follicles. Inhibin A is largely secreted by the corpus luteum, but the serum levels rise in the mid- to late follicular phase and it is suggested that both inhibin A and inhibin B contribute to negative feedback inhibition of FSH in the follicular as well as the luteal phases.[69–72] However, inhibin B serum concentrations in the follicular phase of the human ovulation cycle, whilst showing a similar pattern to that of estradiol, lag somewhat behind those of FSH, suggesting that FSH is regulating inhibin rather than vice versa. The rise of inhibin A in the late follicular phase (at a time when inhibin B is declining) ensures that the net effect of inhibin is maintained.[72] Nevertheless, although it can be argued that inhibins play a role in regulation of FSH, mathematical modeling[73] (Figure 29.6) together with other supportive data (Chapter 28) suggest that estradiol feedback is the principal negative feedback regulator of FSH during the follicular phase of the menstrual cycle in both women with regular ovulatory cycles and those with anovulatory PCOS (discussed further here). However, in older

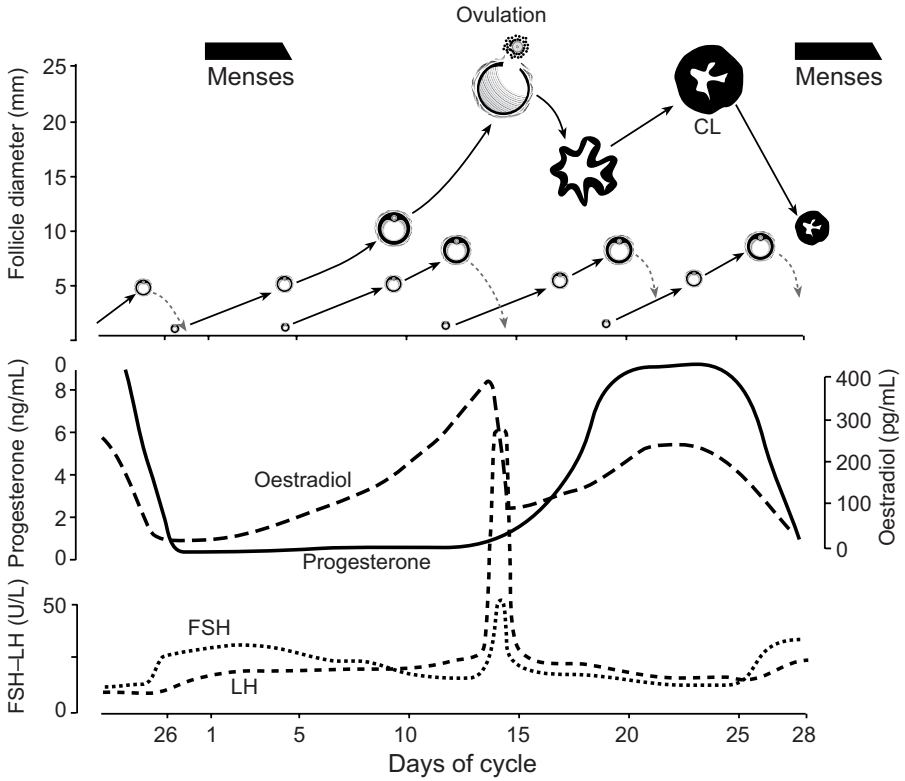

FIGURE 29.5 **The human menstrual cycle.** *Source: From Ref. 66.*

women, elevated FSH levels are associated with normal (or slightly elevated) serum estradiol but lower than normal serum concentrations of inhibin B, and it is feasible that this peptide may play a more important role in regulating FSH secretion in this population.[75]

By the midfollicular phase of the cycle, the dominant follicle has acquired receptors for LH in the granulosa cells, the only time in the life of a follicle that LH receptors are normally present on these cells (this process may be disrupted in PCOS, as shown in the *Polycystic Ovary Syndrome*). In the late follicular phase, the joint action of LH and FSH on the dominant follicle leads to an exponential rise in the levels of estradiol. It is likely that paracrine factors, induced by FSH, also participate in the accelerated growth and increased steroidogenesis in the late follicular phase. For example, the preovulatory follicle shows increased gene expression of type 1 IGF receptors,[76] suggesting that the stimulatory action of endogenous IGFs on estradiol production is amplified at this stage. Indeed, IGF1 has a potent stimulatory action on steroidogenesis in granulosa cells of large antral follicles in the human ovary,[77,78] supporting the notion that local production of IGFs amplifies gonadotropin-induced steroid production in the preovulatory follicle.

The midcycle LH surge is triggered by the rising levels of estradiol and serves three important functions: promoting resumption of meiosis in the oocyte, causing follicular rupture, and stimulating the formation of the corpus luteum. The mechanisms underlying these events are described in great detail elsewhere, as is the function of the corpus luteum (see Chapters 1, 22, and 23). It need only be noted here that among the disorders of the ovulation cycle that are detailed in this chapter, primary dysfunction of the corpus luteum must be considered to be a very rare cause.

DISORDERS OF OVULATION

Disorders of ovulation in women are very common and constitute between 25% and 30% of the causes of infertility. Such disorders also result in menstrual disturbances (amenorrhea, oligomenorrhea (fewer than eight periods per year), or frequent but unpredictably irregular menses) and therefore may cause inconvenience or other morbidities, such as constant vaginal bleeding, in women who are not immediately concerned about their fertility. Disorders of gonadotropin regulation and POI are important causes of anovulation, but PCOS is by far the commonest cause of infrequent or absent ovulation. It is characterized by abnormalities of gonadotropin secretion and also by dysfunctional ovarian follicle development and steroid production. The reproductive and metabolic features of PCOS will therefore be described in considerable detail in this chapter, but due attention will also be paid to other disorders of ovulation in a

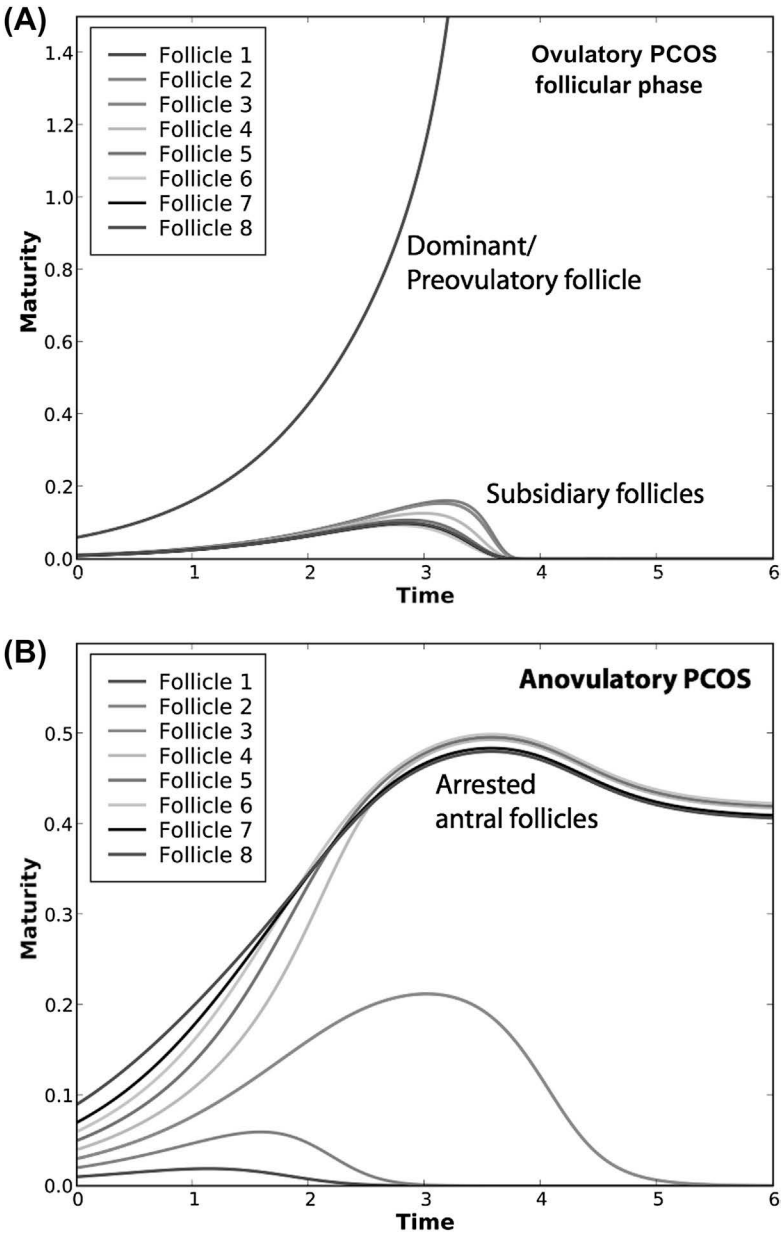

FIGURE 29.6 Mathematical modeling of growth trajectory of follicles in (A) ovulatory and (B) anovulatory polycystic ovary. Time and maturity are in arbitrary units. By applying Lacker's model,[74] it is suggested that in anovulatory PCOS: (1) the primary cause of follicular dysfunction is at the level of the ovary, rather than the pituitary, and is characterized by variable responsiveness of follicles to FSH; (2) arrested follicles have different properties from healthy follicles; (3) the abnormal response to gonadotropins is best characterized by *enhanced* sensitivity to FSH (and LH) in a subpopulation of follicles; and (4) those follicles that are hyperresponsive to gonadotropins produce a high enough concentration of circulating estradiol to suppress FSH to a level that is too low to encourage further development of healthy follicles in the cohort. See also the section Etiology and Pathogenesis of PCOS: Anovulation, Androgen Excess, and Disordered Follicle Development. *Source: Data are from Ref. 73 and Ref. 63.*

systematic fashion. An outline of the causes of anovulation is shown in Table 29.1.

Primary Ovarian Insufficiency

The menopause, defined by the last menstrual period, occurs at an average age of 50.7 years in the Western world.[79] Hypergonadotropic hypogonadism before the age of 40 is most commonly taken to be the definition of POI, which has a prevalence of 1%. It is estimated that for every decade before 40, the prevalence of POI is decreased by a factor of 10. Thus, in the presence of normal karyotype, 1:1000 of women at age 30 has POI, and 1:10,000 at 20, and 1:100,000 of women will present with gonadal failure and primary amenorrhea. POI accounts for 20–50% of females presenting with primary amenorrhea and 10% of those with secondary amenorrhea.[80]

The term POI is preferred to premature ovarian failure as it is an all-encompassing term that accounts for the variable course and occasional remission.[81] The term hypergonadotropic hypogonadism is also used to emphasize ovarian origin whereby the raised concentrations of LH and FSH contrast with the low gonadotropins in hypothalamic or pituitary causes of hypogonadism. Gonadal dysgenesis refers to hypergonadotropic hypogonadism with a known genetic cause such as an abnormal 46XY or 45X karyotype and implies that ovarian development was halted at an early stage of embryonic development. In the case of Turner syndrome, however, it is thought that early ovarian development is usually

TABLE 29.1 Causes of Anovulation

1. Primary ovarian insufficiency		
a. Genetic (e.g., Turner syndrome)		
b. Autoimmune		
c. Irradiation or chemotherapy		
2. Secondary ovarian dysfunction		
a. Disorders of gonadotrophin regulation		
	Organic	– Hyperprolactinemia
		– Kallmann syndrome and its variants
		– Destructive lesions of hypothalamus
	Functional	– Weight loss
		– Exercise
		– Idiopathic
b. Gonadotrophin deficiency		
– Pituitary tumor		
– Pituitary surgery or irradiation		
– Granulomatous or inflammatory infiltration		
3. Polycystic ovary syndrome		

TABLE 29.2 Summary of Pathogenic Mechanisms Causing Primary Ovarian Insufficiency

Genetic	Chromosomal and genetic abnormalities causing abnormal ovarian development or accelerated apoptosis
Autoimmune	Autoimmune ovarian damage is usually presumed because of the association with other organ-specific autoimmune conditions
Metabolic	In galactosemia, abnormal metabolites are thought to mediate ovarian damage
Iatrogenic	Ovarian damage following pelvic surgery and radiotherapy of chemotherapy during treatment of cancer
Environmental factors	Viral infections and toxins such as pesticides are presumed

normal but that the chromosomal anomaly leads to rapid germ cell apoptosis.[82]

Diagnosis of POI

The clinical presentation of POI is variable.[83,84] Very early onset in adolescence leads to pubertal delay, with failure to achieve Tanner breast stage 2 by the age of 13 together with primary amenorrhea. After menarche, the presentation is with symptoms of estrogen deficiency and secondary amenorrhea or as part of a workup for infertility or menstrual disturbance. In addition, POI can be part of rare syndromic conditions that can be genetic or autoimmune in origin.

The diagnosis is based on the finding of elevated serum FSH (>40 IU/L) on at least two occasions separated by a few weeks. The requirement for two samples takes into account the natural history of POI (which can fluctuate) and because the diagnosis is so devastating that certainty is required. While it is the expectation that the condition will usually be permanent, some women follow an unpredictable course of relapse and remission, particularly in the first year after diagnosis.[85] There is a commonly quoted pregnancy rate of approximately 1–5% in women with POI, and it is therefore important to inform women with POI of this phenomenon so that they use contraception when appropriate. Because of this background fertility, case reports of effective treatment of POI must be viewed with caution. Fluctuating ovarian function probably accounts for many cases where the older term "resistant ovary syndrome" was applied, and it is now understood that ovarian biopsy is not predictive of remission (the presence or absence of follicles in the biopsy did not correlate with resumption of ovarian function or chance of pregnancy)[85] and should no longer be included as part of the workup of POI. AMH has found a place as a diagnostic marker of ovarian reserve, but once hypergonadotropic amenorrhea is established it offers little extra information.[86]

A careful family history can identify other affected female members in as many as 30% of cases,[87] and in this situation more extensive genetic screening is indicated and genetic counseling for relatives may be appropriate. In routine practice, the only widely available tests currently available for familial POI are karyotype and fragile X (FRAXA) premutation screening.[88] Karyotype assessment should be considered in those with a family history or unusually early onset of POI, and FRAXA should be offered to all women with POI because of its value in detecting a new pedigree at risk of fragile X syndrome.

The etiology of POI is diverse, with multiple mechanisms leading to the same clinical picture.[89] Table 29.2 shows the main categories of pathogenic mechanisms that lead to ovarian insufficiency. Despite diagnostic advances, particularly in the identification of genes causing POI, there remains a large proportion of cases for whom the etiology is unknown.[84]

Pathogenesis of POI

GENETIC CAUSES OF POI

A genetic etiology for POI is suggested not only by a positive family history, which may be present in 30% of cases, but also if there are features of an associated syndrome (Table 29.3). Cytogenetic chromosome anomalies occur in about 2% of cases, with the majority involving the X chromosome.[90–92]

X chromosome defects and Turner syndrome: Defects of the X chromosome associated with POI include complete or partial deletion of one X (Turner syndrome),

TABLE 29.3 Genetic Syndromes Associated with Primary Ovarian Failure

Syndrome	Gene Locus
Autoimmune polyendocrinopathy type I	*AIRE*
Blepharophimosis, epicanthus inversus, and ptosis type 1 (BPES)	*FOXL2*
Denys–Drash syndrome	*WT1*
Fragile X associated syndromes	*FMR1*
Galactossemia	*GALT*
Malouf syndrome	*LMNA*
Mulibrey nanism	*TRIM37*
Perraults syndrome type 1 and 2	*HSD17B4, HARS2*
Progressive external ophthalmoplegia	*POLG*

trisomy X, or X-autosome translocations. In the case of Turner syndrome variants, there is a great deal of variability in severity of the condition, and this corresponds approximately to genotype and the amount of X chromosome disruption. Ovarian histology can vary from streak ovaries to those with well-preserved ovarian structure with numerous follicles. Those with monosomy X (45X) tend to be the most severely affected, while those with partial deletions of the X chromosome or mosaic 45X or 46XX karyotype often lack the typical phenotypic features of the syndrome but may present with only ovarian insufficiency and secondary amenorrhea. Amongst women with Turner syndrome, the prevalence of ovarian failure is between 80% and 90%, with most associated with primary amenorrhea and complete pubertal delay.

X chromosome deletions appear to segregate into two specific regions: POI1 at Xq26-qter and POI2 at Xq13.3–Xq21.1.[93,94] There are various proposed mechanisms by which X chromosome defects cause POI. Genes responsible for ovary development and/or oogenesis located along these critical regions may be interrupted, although breakpoint genes that determine ovarian function have not been convincingly identified. That is, although genes disrupted by breakpoints have been identified in women with POI, these have not subsequently been shown to necessarily be causal mutations by screening affected populations.[90] Many breakpoints on the X chromosome occur in regions where known genes are scarce, leading to the possibility that long-range position effects on the expression of flanking genes account for the adverse effect on ovarian function. It seems most likely, however, that structural rearrangements of the X chromosome do not affect single genes but rather disrupt normal pairing at meiosis, leading to meiotic arrest and subsequent follicle atresia. It must also be noted, however, that some X chromosome breakpoints are not associated with POI.[94]

Single-gene defects causing POI: A growing number of genes have been linked to ovarian failure, although the strength of evidence linking each anomaly with POI is variable.[95] The most clinically useful genetic association is that between carriers of the fragile X premutation and ovarian failure. Premutations in the *FMR1* (fragile X and mental retardation) gene occur in 3% of women with sporadic POI and 15% of those with the familial form, and screening for mutations of this gene is all that is currently advised in the United Kingdom as part of a routine workup of POI.[96] In general, the phenotype of this subgroup of women with POI is indistinguishable from others, although a minority is reported to suffer a progressive neurological deficit in the form of an intention tremor. The pathway by which *FMR1* premutations damage ovarian function is unclear as the described variants of this gene (notably expansion of exon 1) are not thought to affect protein transcription, even though the protein is expressed within the ovary. It is probable that noncoding RNAs are responsible for the molecular pathogenesis.[91]

AUTOIMMUNE CAUSES OF POI

Autoimmune mechanisms may be involved in pathogenesis of up to 30% of cases of POI.[97] POI has been reported to be associated with various endocrine (thyroid, adrenal, hypoparathyroidism, diabetes mellitus, and hypophysitis) and nonendocrine autoimmune disorders (chronic candidiasis, idiopathic thrombocytopenic purpura, vitiligo, alopecia, autoimmune hemolytic anemia, pernicious anemia, systemic lupus erythematosus (SLE), rheumatoid arthritis, Crohn's disease, Sjogren's syndrome, myasthenia gravis, primary biliary cirrhosis, and chronic active hepatitis).[98,99] In many cases, nonovarian autoimmune involvement exists only at subclinical levels.[100,101] POI may be part of the autoimmune polyglandular syndromes (APSs) when accompanied by other autoimmune endocrinopathies. POI is more common with APS types I and III than with APS type II. The single most common autoimmune association is with hypothyroidism, which occurs in 10% of women with POI.

Various pathways of autoimmune ovarian damage have been described, but a reliable ovarian specific autoimmune marker is still lacking. Several putative pathogenic autoantibodies have been explored.[102] Antiovarian antibodies detected by routine *immunofluorescence* have been reported in several studies of women with POI, but their specificity and pathogenic roles are questionable. The incidence of antiovarian antibodies in POI in different studies has been reported to vary from 4% to 69%.[103] Other candidate autoantibodies include those directed against steroidogenic enzymes (such as 3β-hydroxysteroid dehydrogenase), gonadotropins and their receptors, the corpus luteum, the zona pellucida,

and oocytes. None of these, however, have been validated as a prime mover in the process or found to be useful as a diagnostic marker of autoimmune ovarian failure.[104] Therefore, in the clinical workup of POI, screening for an autoimmune etiology is usually only possible by seeking coexisting autoimmune diseases. One report has shown raised serum inhibin to be a distinguishing feature of autoimmune POI compared to other forms, but whether this will be sufficiently robust to be clinically useful is not yet clear.[105]

An animal model of autoimmune oophoritis that develops in mice after neonatal thymectomy has helped in understanding the potential pathogenic mechanisms of autoimmune POI and ovarian antigens.[106] Animal and human disease both show a similar histological distribution of ovarian lymphocytic infiltration, the production of antiovarian autoantibodies, and a reduced natural killer cell activity. In the mouse model, altered T cell regulation has been implicated in the pathogenesis of ovarian failure, and cells with a T helper phenotype can mediate the oophoritis.

Women with idiopathic POI show an increased number of activated T cells in peripheral blood. Similar findings have been described in other autoimmune endocrinopathies, such as recent-onset Graves' disease, type 1 diabetes mellitus, and Addison's disease. However, postmenopausal women also show raised numbers of activated peripheral T cells, and estrogen substitution has been shown to lower the number of activated peripheral T cells in women with POI. Therefore, it is difficult to be certain whether the raised numbers of activated blood T cells are the cause or the result of ovarian failure in this situation.[98]

MISCELLANEOUS CAUSES OF POI

Viral oophoritis is a possible occult etiology that could theoretically account for the many cases of idiopathic POI. There is, however, little direct evidence of viral ovarian damage beyond case reports such as mumps oophoritis.[107] Cigarette smoking is known to be associated with earlier age of natural menopause, but whether environmental effects are sufficiently strong to cause POI is unlikely. The available data regarding the effects of endocrine disruptors, heavy metals, solvents, pesticides, plastics, and industrial chemicals on female reproduction are equivocal.[108]

Management of POI

The major medical issues for health surveillance in women with POI revolve around the quality of life and bone protection offered by hormone replacement therapy (HRT). Options for reproduction include oocyte donation, but adoption should not be overlooked. Women also require personal and emotional support to deal with the impact of diagnosis on their health and relationships. Long-term follow-up is essential to monitor HRT and to consider emerging associated autoimmune pathology.

Deficiency and Disordered Regulation of the Gonadotropins

Abnormalities of gonadotropin secretion account for about 30% of women with either oligo- or anovulation and are found in over 50% of those who present with amenorrhea. Whereas primary pituitary deficiency of gonadotropins is rare, abnormal hypothalamic regulation of gonadotropin secretion is common, whether functional (in which there is no structural pathology—the most frequent form) or organic in origin. In recent years, the discovery of "new" peptides such as kisspeptin, which have an important role in the afferent input into the GnRH neuronal network, has added to our increasing understanding of how the hypothalamic regulation of gonadotropin regulation is subject to influence by environmental factors, particularly those related to energy balance.

Specific Organic Disorders of Gonadotropin Secretion

Organic causes of gonadotropin deficiency (with the exception of hyperprolactinemia) are relatively uncommon but important to recognize. The underlying reasons include specific deletions or modifications of the genes encoding key peptides in the hypothalamic regulation of gonadotropin synthesis and secretion, and destructive lesions of the pituitary or hypothalamus. A comprehensive list of hypothalamic causes of anovulation is shown in Table 29.4. The causes include developmental abnormalities and destructive granulomatous or primary neoplastic lesions, as well as (rarely) secondary deposits of systemic neoplasia.[89] There is often, in such cases, evidence of deficiency of other pituitary hormones, including growth hormone, thyrotropin, and adrenocorticotropic hormone. There may also be involvement of the neurohypophysis, and the consequent, additional presentation with diabetes insipidus is a clear indication of a destructive hypothalamic lesion.

KALLMANN SYNDROME AND ITS VARIANTS

There has been an explosion of knowledge about Kallmann syndrome and its variants, particularly in the last decade when increasing sophistication of genetic analyses uncovered numerous susceptibility loci as a major etiological factor in congenital aberrations in the GnRH neuronal network. Indeed, there is recent evidence that there may be a genetic predisposition to functional disorders of gonadotropin secretion related to weight loss, stress, or exercise, as discussed in this section. Although genetic causes of GnRH deficiency are more common in males than in females, they still represent an important

TABLE 29.4 Hypothalamic Lesions That Can Result in Decreased GnRH Secretion and Amenorrhea

Developmental abnormalities cysts
Craniopharyngioma (occasionally intrasellar location)
Germinoma
Hamartoma
Chordoma
Epidermoid and dermoid
Primary tumors of the central nervous system
Perisellar meningioma
Optic glioma
Ependymoma
Malignant and systemic diseases of the central nervous system
Hodgkin's disease
Non-Hodgkin lymphoma
Leukemic infiltration
Histiocystosis
Eosinophilic granuloma
Giant cell granuloma (tumor)
Granulomatous diseases
Neurosarcoidosis
Wegener's granulomatosis
Tuberculoma
Syphilis

From Ref. 89.

subgroup in the etiology of delayed puberty in girls and in hypothalamic amenorrhea in adults.

The classic presentation of Kallmann syndrome is the association of hypogonadotropic hypogonadism with anosmia. With the knowledge that Kallmann syndrome was associated with an X chromosome deletion at Xp22.3, Pfaff and colleagues, in a study of fetal tissue carrying that mutation, uncovered the mystery of the link between these features.[109] GnRH neurons were found to originate in the olfactory placode, and later studies in the rodent revealed that these neurons migrate during fetal life, together with the olfactory neurons, toward the medial basal hypothalamus. This process is a complex one and involves several possible mechanisms[110,111] that include a defect in the neural cell adhesion molecule (NCAM) that provides a "scaffold" for migration of both GnRH neurons and the olfactory nerve from the olfactory placode.[112] The Kallmann gene *KAL1* was later found to code for anosmin 1, an extracellular matrix protein implicated in neuronal development and migration.[113]

Since the identification of *KAL1* mutations as a cause of genetic GnRH deficiency, there has been steady progress in understanding the genetic origins of hypogonadotropic hypogonadism, knowledge of which has accelerated impressively in the last decade.[114] This has been an iterative process in which the increasing understanding of the biology of afferent pathways controlling GnRH neuronal secretion has informed genetic studies and in which the finding of novel mutations in patients with GnRH deficiency has led to the discovery of new ligands and their receptors that are functionally important in normal GnRH secretion. Genetic causes of GnRH deficiency can conveniently be categorized according to their developmental origin as (1) disorders of the development and migration of GnRH neurons, or (2) disorders of homeostasis and GnRH deficiency.[114] Of the novel peptides that have been shown to contribute to a functionally important afferent input into the GnRH neural network, it is kisspeptin that has provided one of the most intriguing stories of discovery.[115] The kisspeptin receptor is a G protein-coupled receptor (GPR), and it was the receptor GPR54 that was discovered (by investigators in the cancer field) before the ligand was identified. Targeted deletions of *GPR54* in mice were subsequently shown to cause failure to enter puberty and, around the same time, mutations in *GPR54* were found to be associated with hypogonadotropic hypogonadism in humans.[116,117] Kisspeptin has a key role in the normal function of GnRH secretion, and its therapeutic applications in management of hypogonadotropic hypogonadism are being pursued by several groups.[118] Administration of exogenous kisspeptin increases gonadotropin secretion and may yet prove to be an important means of induction of ovulation in women with hypothalamic amenorrhea (HA). Of particular interest is the observation that a continuous low-dose infusion of kisspeptin10 (a truncated form of the peptide) is capable of producing LH pulses in healthy young males,[119] although it is not yet clear whether, in either males or females, a normal pulsatile pattern can be achieved by such means. Clearly, this observation as well as more recent studies in healthy females raise the possibility of the application of kisspeptin analogs in promoting pulsatile and cyclical gonadotropin secretion in women with hypogonadotropic hypogonadism.[120]

Other key peptides (or their receptors) in the regulatory pathway of GnRH pulse generation include neurokinins and their receptors. Mutations in the neurokinin B gene *TAC3* and its receptor *TACR3* have been found in cases of familial hypogonadotropic hypogonadism.[121] Genetic mutations in genes coding for GnRH itself (*GnRH1*), the GnRH receptor (*GNRHR*), fibroblast growth factor 8 (*FGF8*), and FGF receptor 1 (*FGFR1*), as well as in genes controlling multiple functions during development (e.g., *DAX1*), have also been reported.

Patients with *DAX1* mutations present with adrenal insufficiency together with hypogonadotropic hypogonadism. This rare syndrome is even less common in females but has been reported as a cause of hypogonadism in girls.[122]

PITUITARY TUMORS AND OVARIAN DYSFUNCTION

Generally speaking, hormone-secreting pituitary tumors are amongst the least common causes of disordered ovulation, but the one notable exception is the prolactin-secreting tumor, which is the commonest functional pituitary tumor in both men and women.[123] Indeed, hyperprolactinemia is one of the most common causes of amenorrhea, accounting for upward of 10% of cases.[29] The pathogenesis, presenting features, and diagnostic approach will therefore be described in some detail in the section Hyperprolactinemia, and its management in the discussion of hyperprolactinemia in the section Clinical Management of Ovulatory Disorders. Other pituitary tumors such as somatotrope and corticotrope adenomas are rare causes of anovulation.[124,125]

HYPERPROLACTINEMIA

Hyperprolactinemia has a variety of causes, the most clinically important of which is a prolactin-secreting pituitary tumor (prolactinoma).[123,126] Raised serum prolactin levels are not necessarily pathological; there are physiological variations of prolactin, which include a sleep-related rise that can confound the results of a prolactin measurement taken in an early-morning clinic. Other physiological stimuli are, of course, pregnancy and lactation. Hyperprolactinemia may be a feature of primary hypothyroidism, and so thyroid function tests are important in women with elevated prolactin levels. A variety of pharmaceutical agents can be associated with elevation of serum prolactin, most notably dopamine antagonists including psychotropic drugs such as phenothiazines.[123]

Persistently elevated serum concentrations of prolactin are a common and long-recognized cause of amenorrhea and anovulation (Table 29.1). The syndrome of amenorrhea with galactorrhea (inappropriate lactation) had been reported for many years before the advent of a reliable radioimmunoassay for prolactin in the early 1970s. The development of such assays closely followed the identification of prolactin as a distinct hormone from growth hormone. Until then, these so-called lactogenic hormones were difficult to distinguish from each other biochemically (see the introduction). The application of immunoassays revealed that hyperprolactinemia accounted for upward of 10% of causes of amenorrhea.[29] It was clear that in many cases of hyperprolactinemia, there was radiological evidence of a pituitary tumor. With subsequent improvement in imaging techniques, it is estimated that around 50% of women with hyperprolactinemia have a visible prolactinoma, the majority measuring less than 10 mm in diameter and termed microprolactinomas.

The key endocrine features of hyperprolactinemic amenorrhea are estrogen deficiency associated with low or normal gonadotropin levels.[127] This profile is usually indicative of a hypothalamic disorder of gonadotropin regulation, and indeed this appears to be the major mechanism underlying the failure of ovulation. That hypersecretion of prolactin impacts the GnRH pulse generator (rather, by exerting effects at the level of the pituitary or ovary) is suggested by aberrant pulsatile gonadotropin secretion and borne out by the finding that the administration of exogenous, pulsed GnRH results in ovulatory cycles in the face of persistent hyperprolactinemia.[128]

Functional Disorders of Gonadotropin Regulation

Functional hypothalamic amenorrhea (FHA) is discussed in Chapter 36 but will be briefly considered here. Functional disorders of gonadotropin regulation include women with weight loss-related or exercise-related amenorrhea in which the common feature is energy deficiency associated with low body-fat composition. Conversely, obesity may be associated with reproductive dysfunction, but the adverse effect of obesity on menstrual cyclicity is affected predominantly by an interaction with PCOS (see the section Polycystic Ovary Syndrome). The patterns of pulsatile gonadotropin in weight loss-related amenorrhea may vary according to the severity of the disorder (Figure 29.7). There is also an important group of women with idiopathic hypothalamic hypogonadism in whom changes in body composition are less evident but who often manifest a psychological profile that is suggestive of a stressful lifestyle.[129] Although women with FHA have no organic cause for their hypothalamic dysfunction, it is clear that other elements of the endocrine system may be disturbed, including particularly the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–thyroid axes.[129] Berga has suggested that a common feature in the etiology of FHA is stress, both psychological and metabolic.[129–131] Even in women with FHA, there may be a genetic predisposition to develop disordered gonadotropin secretion.[132]

Anovulation and amenorrhea associated with exercise are yet another example of the complex interaction of environmental influences on the HPO axis.[131,133] Excessive exercise, for example amongst competitive, long-distance athletes,[134] may in itself be responsible for the energy deficiency that results in compromised GnRH secretion. However, psychogenic factors may also have an important role in amplifying the effects of exercise.[129,131]

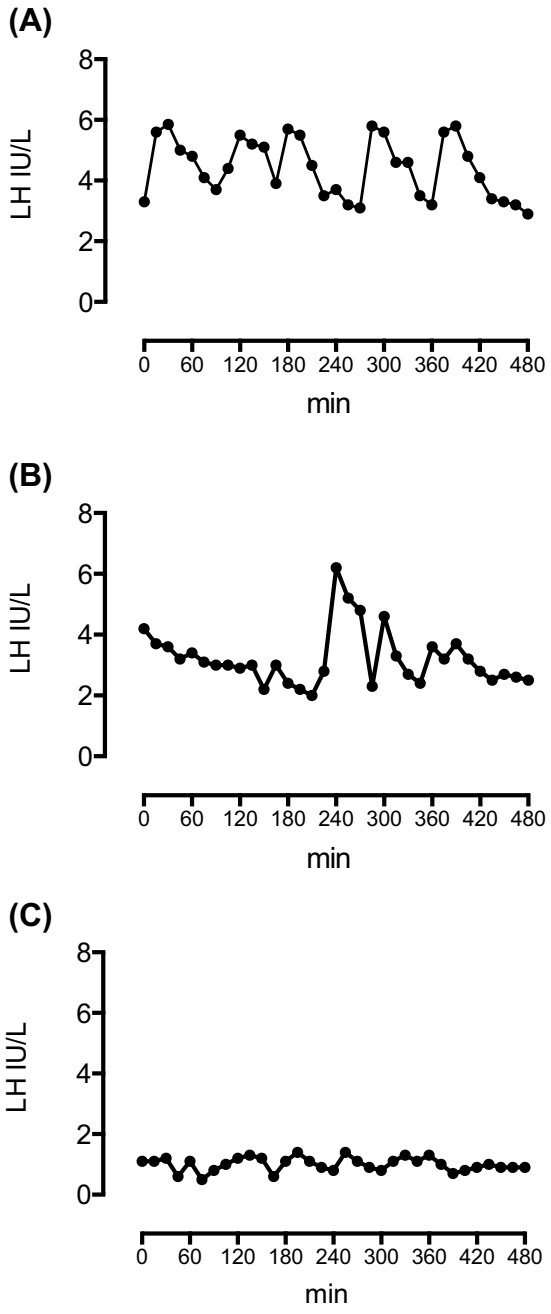

FIGURE 29.7 LH secretory patterns in (A) a normal woman with regular menses, studied in the midfollicular phase; (B) a woman with partially recovered weight loss-related amenorrhea; and (C) a woman with a persistently low BMI.

Polycystic Ovary Syndrome

PCOS is the commonest endocrine disorder in women, and it is now recognized that not only is PCOS the most frequent cause of anovulatory infertility and of hirsutism, but also it is associated with characteristic metabolic abnormalities that may carry an increased risk, in the long term, for the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease. However, the etiology remains uncertain. The heterogeneity of the clinical presentation has inevitably raised the question as to whether PCOS is one disorder or whether its origins are as variable as its manner of presentation. There remain critical questions and controversies not only regarding the etiology of the syndrome but also in diagnostic criteria, investigation, and management of this complex disorder.

Clinical Aspects of PCOS

DEFINITION AND DIAGNOSIS OF PCOS

There has always been debate about the definition of PCOS, and it seems likely to remain so until the specific cause or causes of the syndrome can be elucidated. The classic definition, arising from the National Institute for Child Health and Human Development (NIHCD)- sponsored conference on PCOS in 1990, includes the clinical manifestations of anovulation and hyperandrogenism in women[135]; obesity is a common but not universal accompaniment. Typically, but not exclusively, these features are associated with hypersecretion of LH and androgens but with normal, or slightly low, serum concentrations of FSH. In recent years, the ability to identify PCOS by pelvic ultrasonography (because of multiple antral follicles and increased ovarian stroma) has provided new insight into the spectrum of clinical and biochemical presentation of women with a polycystic morphology of the ovaries.[136,137] It is now evident that this spectrum includes both anovulatory women who are without hirsutism (but are usually hyperandrogenemic) and, conversely, those who are hirsute and have regular ovulatory cycles (but may also have elevated serum LH concentrations). Thus, irrespective of clinical presentation, there is a typical pattern of biochemical abnormalities that seems to unite these subgroups of women with ultrasound evidence of polycystic ovaries.

The recognition that the spectrum of presentation of women with PCOS is broader than was first thought has led to a revision of the diagnostic criteria for PCOS. The joint European Society for Human Reproduction and Embryology–American Society for Reproductive Medicine (ESHRE–ASRM) consensus meeting on PCOS, held in Rotterdam in May 2003, resulted in the recommendation that the definition of PCOS be revised to encompass women with hyperandrogenism and polycystic ovaries but who had regular cycles (see Table 29.5). This definition, like the classic definition, excludes patients who have polycystic ovaries on ultrasound but in whom the primary diagnosis is of pituitary or adrenal diseases (e.g., hyperprolactinemia, acromegaly, and classical or nonclassical congenital adrenal hyperplasia). To add to this definition, the Androgen Excess and PCOS Society (AE-PCOS) suggested a set of diagnostic criteria that was essentially a compromise between the "NICHD" and "Rotterdam" criteria, in that it includes women

TABLE 29.5 Diagnostic Criteria for PCOS

NIH 1990	Rotterdam 2003	AE-PCOS Society 2006
1. Chronic anovulation 2. Clinical and/or biochemical signs of hyperandrogenism (with exclusion of other etiologies, e.g., congenital adrenal hyperplasia) *(Both criteria needed)*	1. Oligo- and/or an-ovulation 2. Clinical and/or biochemical signs of hyperandro genism 3. Polycystic ovaries *(Two of three criteria needed)*	1. Clinical and/or biochemical signs of hyperandrogenism 2. Ovarian dysfunction (oligo-an-ovulation and/or polycystic ovarian morphology) *(Both criteria needed)*

with hyperandrogenism and regular cycles but excludes women with oligomenorrhea without clear evidence of androgen excess.[138] Despite some inconsistencies in results between groups (that may be attributed to different methods of diagnosis), comparative studies have demonstrated that there is a high degree of concordance of ultrasound with histological features of polycystic ovaries and with clinical and endocrine evidence of the syndrome.

The report of the recent NIH Office of Disease Prevention Evidence-based Methodology Workshop on Polycystic Ovary Syndrome recommended that the broader diagnostic criteria were the more generally applicable and practical (http://prevention.nih.gov/workshops/2012/pcos/resources.aspx).

PREVALENCE AND PRESENTATION OF PCOS

PCOS is a highly prevalent disorder. The "classic" syndrome (i.e., the combination of oligo-amenorrhea and androgen excess) may be found in more than 5% of women of reproductive age. Using ultrasonographic, clinical, and endocrine criteria, it has been estimated that PCOS accounts for about 30% of women presenting with secondary amenorrhea, more than 80% of women with oligomenorrhea, and, perhaps most surprisingly, between 60% and 90% of women with hirsutism but who have regular menses. PCOS accounts for 75–85% of cases of anovulatory infertility. The prevalence of obesity in women with PCOS varies from 35–40% to over 70%. Its presence influences the clinical presentation of women with polycystic ovaries; hirsutism and menstrual disturbances are significantly more common in obese compared with lean subjects.

The characteristic endocrinopathy of raised mean levels of serum androgens and LH has been reported in all published series of women with PCOS, but the variation in LH levels between individuals (which depends on not only the criteria for diagnosis but also the method of measurement) means that the diagnostic value of a single hormone measurement is limited. The heterogeneity of LH is evident even when evaluating episodic release of LH (Figure 29.8). Similarly, although serum concentrations of testosterone and androstenedione are elevated in women with PCOS, there is a good deal of variation from patient to patient. This is illustrated by the observation that anovulatory but nonhirsute women with polycystic ovaries may have hyperandrogenemia whilst hirsute women with polycystic ovaries may have normal serum androgen levels. This phenomenon may be explained by the fact that both production and clearance of androgens are increased in women with hirsutism.[139] Low serum concentrations of sex hormone-binding globulin (SHBG)—the principal transport protein for testosterone in the blood—may contribute to increased clearance of testosterone. Blood levels of SHBG are inversely correlated with body mass index (BMI),[140] which may help to explain why obese subjects with PCOS are more likely to be hirsute than their lean counterparts, despite similar serum levels of testosterone. The effects of obesity on SHBG are likely to be mediated by insulin.[141,142] The relevance of hyperinsulinemia and insulin resistance to PCOS is discussed in this chapter.

Other hormonal abnormalities, including hyperprolactinemia and impaired secretion of growth hormone, have been described in women with PCOS. Moderate hyperprolactinemia occurs in 5–10% of women with typical clinical and biochemical features of PCOS.[143,144] The significance of hyperprolactinemia in PCOS remains unclear. Impaired growth hormone secretion in women with polycystic ovaries may simply be a function of accompanying obesity rather than a specific feature of the syndrome.

METABOLIC ABNORMALITIES AND THEIR CLINICAL CONSEQUENCES IN WOMEN WITH POLYCYSTIC OVARIES

The reproductive consequences of PCOS have been recognized for several decades, but over the last 25 years there has been an increasing number of studies illustrating that the syndrome is also associated with a characteristic metabolic disorder.[143,145,146] This, in turn, has led to concern about the impact of PCOS on long-term health, particularly with regard to the advent of diabetes and coronary heart disease.[147] The central features of the metabolic disturbance are peripheral insulin resistance and hyperinsulinemia.[148] The mechanism of these disturbances is not certain, but there is evidence for intrinsic abnormalities of both insulin action and pancreatic beta cell function.[146] There is an important interaction of polycystic ovaries with body weight so that whilst lean women with PCOS often have normal insulin levels and sensitivity, the majority of those whose BMI is greater than 30 kg/m^2 have reduced insulin sensitivity compared with weight-matched controls.[149,150] It is not surprising, therefore, that obese young women with

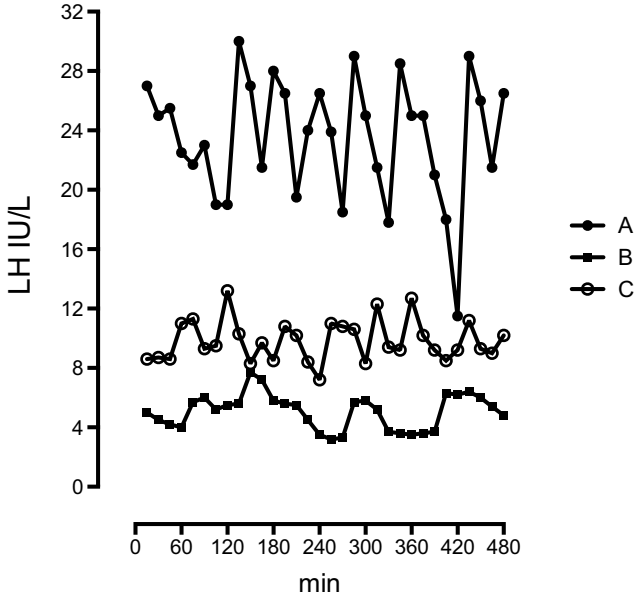

FIGURE 29.8 Heterogeneity of pulsatile LH secretion in women with PCOS showing (A) elevated mean LH and high-amplitude pulses; (B) levels of LH that fall into the normal range (2–12 U/L) (although still above the mean level in normal subjects), with occasional high-amplitude pulses; and (C) normal mean LH and normal pulse amplitude.

PCOS have a high prevalence of impaired glucose tolerance (and indeed, some may have frank diabetes), and there seems little doubt that women with PCOS have a significantly increased risk of developing T2DM in later life.[151] As yet, long-term follow-up studies have been few and far between, but in one epidemiological analysis of a large cohort of women with a history of PCOS (mean age: 57 years), the relative risk of T2DM was found to be three times that of the reference population.[152] Abnormalities of lipids and lipoproteins have also been widely reported in women with PCOS, but it remains uncertain whether these are independent of the effects of obesity.[153]

These metabolic features, together with centripetal fat distribution, constitute a cluster of risk factors for cardiovascular disease, and this has been a major concern in considering the long-term management of patients with PCOS.[147] However, interpretation of the significance of the metabolic abnormalities in terms of cardiovascular risk is by no means easy. First, there are inconsistencies between studies in the features of the dyslipidemia; and, second, it is not yet clear how the combined risk factors translate into a real risk of developing cardiovascular disease.

The remaining uncertainties surrounding the reports of the dyslipidemia of PCOS emphasize the importance of counseling caution about pronouncing on the implications for cardiovascular health of a diagnosis of PCOS in young women. Whilst there seems little doubt that PCOS, independently of obesity, constitutes a significant risk factor for T2DM, there is, at present, no direct evidence for increased morbidity or mortality from coronary heart disease. Endothelial dysfunction and ultrasound evidence of carotid intima-medial abnormalities have been reported in young and middle-aged women with PCOS,[154,155] but these must still be regarded as surrogate indices of cardiovascular disease. Epidemiological data available to date provide no conclusive evidence of an increase in morbidity or mortality from coronary heart disease in a population of middle-aged or elderly women with a history of PCOS.[156,157] However, it is quite conceivable that the incidence of coronary heart disease will diverge from normal as this cohort of middle-aged women grows older. Further long-term follow-up studies are required before a definitive link can be established. It has already been demonstrated that lifestyle changes (especially in diet) can markedly improve the metabolic profile of obese women with PCOS[158–160] and, at the very least, can be expected to reduce the chance of developing T2DM. Metformin and other insulin-sensitizing agents also have a part to play in limiting the impact of the metabolic complications of PCOS.[147,161]

Etiology and Pathogenesis of PCOS: Developmental, Genetic, and Environmental Factors

The precise etiology of PCOS remains uncertain, but there is now a wealth of data from both human studies and animal models that has brought a new understanding of genetic and environmental factors that have a major impact on the genesis of PCOS. This knowledge has progressed hand in hand with new insights into the mechanisms of anovulation and the nature of the metabolic disturbances that are characteristic of the syndrome.

EVIDENCE FOR DEVELOPMENTAL ORIGINS OF PCOS

It has been proposed that PCOS has its origins in early life and that the polycystic ovary is genetically predisposed to hypersecrete androgens, certainly at puberty but also in the early postnatal period or even during fetal development[162] (Figure 29.9). It is very unlikely that maternal androgen excess affects fetal exposure to androgen because high circulating concentrations of SHBG and placental aromatase activity provide an effective barrier between mother and fetus.[162,163] The dogma has been that the fetal ovary is not steroidogenically active, but recent data indicate that somatic cells in the human fetal ovary clearly express CYP17A1 (17-α-hydroxylase/17–20 lyase), the key enzyme in androgen biosynthesis.[164]

Extrapolating from studies in the prenatally androgenized (PA) rhesus monkey or sheep, the hypothesis is that increased exposure to androgens during development leads to the abnormalities of endocrine and metabolic

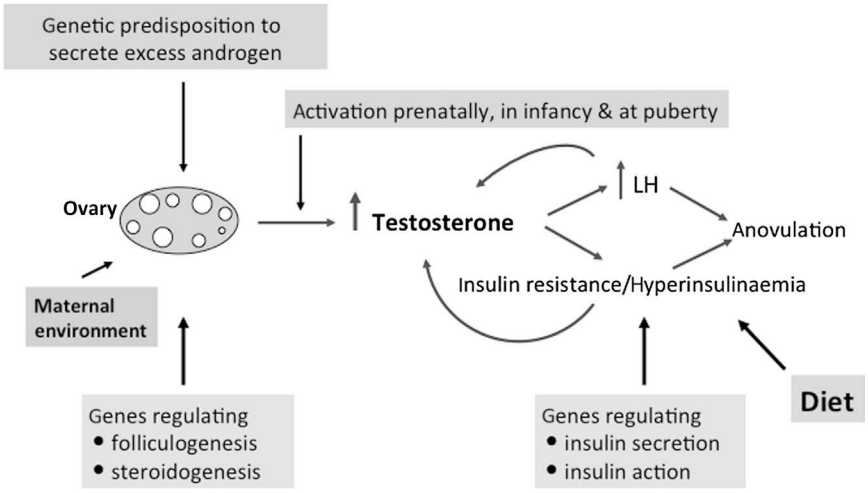

FIGURE 29.9 Proposed developmental etiology of polycystic ovary syndrome (PCOS).[162,163] It is suggested that the ovary is genetically predisposed to hypersecrete androgens, perhaps as early as intrauterine life but certainly during the activation of the hypothalamic–pituitary–ovarian axis that occurs transiently in infancy and in a sustained manner at puberty. Higher than normal circulating levels of testosterone "program" the hypothalamic–pituitary unit to produce high tonic levels of luteinizing hormone (LH), and also amplify the physiological insulin resistance of puberty. Higher than normal concentrations of LH and insulin further enhance ovarian androgen production and may contribute to the mechanism of anovulation.

function that are characteristic of PCOS. Indeed, both the PA rhesus monkey and the PA sheep have proved to be instructive models in understanding the development of PCOS in humans, and have pointed to a key role for androgens in the etiology of both reproductive and metabolic manifestations of this disorder. The PA rhesus monkey, during adulthood, displays abnormal ovarian morphology, ovarian hyperandrogenism (and adrenal androgen excess), hypersecretion of LH, insulin resistance, and anovulation in relation to increased body weight.[165–168] Studies to date in the PA sheep have yielded similar findings with respect to reproductive and metabolic sequelae.[169]

If it is reasonable to hypothesize that genetically regulated androgen excess has a key role in the etiology of PCOS, it is nevertheless important to consider the effects of environmental factors, particularly nutritional influences (Figure 29.9). Prenatally, whereas maternal androgens are unlikely to affect the fetus for the reasons stated here, it remains entirely possible that the metabolic status of the mother does have an impact, for example in the case of maternal diabetes or simple obesity. Certainly, in the postnatal life of the adolescent girl or adult with symptoms of PCOS, diet can have a profound influence on phenotype (as discussed in this chapter).

EVIDENCE FOR A GENETIC BASIS OF PCOS

PCOS is more common within families than in the general population, it has a greater concordance between identical twins than dizygotic twins, and there is evidence for heritability of biochemical traits. These features provide strong evidence for major genetic influence in the etiology of PCOS.[158,166] Given the heterogeneity of PCOS, it has always seemed likely that the genetic basis of PCOS, as in other complex endocrine traits (such as T2DM), involves the interaction of small effects contributed by polymorphisms at several susceptibility loci. The very diversity of presenting features of PCOS raises the prospect of several candidate biochemical pathways to explore in searching for genes involved in the etiology of PCOS. Thus, genes implicated in ovarian follicle development (e.g., those in the TGFβ family), genes involved in the androgen biosynthetic pathway (e.g., *CYP11A*, *CYP17A1*, and *CYP19*), and those affecting insulin secretion or action (e.g., transcription factor 7-like 2 (*TCF7L2*); potassium inwardly rectifying channel, subfamily J, member 11 (*KCNJ11*); insulin receptor; and peroxisome proliferator-activated receptor gamma (*PPARγ*)) have all been cited as plausible candidates. But despite the exploration of more than 150 candidate genes, this approach has, so far, yielded disappointing results. Findings have often been ambiguous because of relatively small sample sizes (i.e., seriously underpowered) and because of genetic and phenotypic heterogeneity within a study population. Importantly, very few of the candidate loci that have been implicated have been confirmed by replication in a subsequent and separate population.[170,171]

There is, however, good evidence for a role in PCOS for the genes coding for fibrillin 3 (FBN3) and the fat mass- and obesity-associated (*FTO*) genes. Evidence for linkage disequilibrium was observed at D19S884, a dinucleotide repeat marker on chromosome 19p13.2.[172–174] Genotyping of the region around D19S884 subsequently identified the *FBN3* gene as a likely candidate for the PCOS susceptibility gene. The association of the *FTO* locus (on chromosome 16) and T2DM and obesity led researchers to explore a role for this gene in the etiology of PCOS. In a case-control study, an association was found between PCOS status and *FTO* genotype.[175] *FTO* variants that were known to predispose to obesity were also implicated in altered susceptibility to PCOS, confirming the mechanistic link between these conditions. Confirmation of the association between *FTO* and PCOS has been provided in studies of other populations[176] and results of a recent meta-analysis support the view

that variation at the *FTO* locus contributes to the etiology of PCOS. Although its role in the genetic makeup of PCOS is predominantly by increasing the susceptibility to obesity (and therefore "amplifying" the phenotype of PCOS), there is limited evidence that polymorphisms in *FTO* contribute to the etiology of PCOS by a mechanism that is independent of body fat mass.[175]

In summary, the candidate gene approach to PCOS has been largely unproductive and, researchers have, not surprisingly, turned their attention to genome-wide association studies (GWAS) of the kind that has paid dividends in the search for genes in T2DM. Although this requires considerable resources in the study of very large case-control populations, early studies have already proved informative. The first reported GWAS identified susceptibility loci for PCOS on chromosome 2p16.3, 2p21, and 9q33.3 in a population of 744 Han Chinese women, and although this was a relatively small sample for a GWAS (compared, e.g., with those employed in studies of T2DM genes), results were replicated in two further populations of Han Chinese[177] and subsequently in other large Chinese cohorts.[178,179] Further evidence implicating the chromosome 2p16.3 locus in the etiology of PCOS comes from a replication series of women of European ancestry.[180] The significance of this locus is that this is the region of DNA in which genes coding for gonadotropin receptors *FSHR* and *LHCGR* are found. Other reproducible variants that were identified initially in the Chinese studies were at the loci for DENND1A (Differentially expressed in normal and neoplastic cells (DENN) domain-containing protein 1A; associated with Rab function in membrane trafficking) and THADA (thyroid-associated protein). Significantly, these findings have been reproduced in recent studies of populations of European origin, suggesting that these pathways may play a part in the genetic origin of PCOS.

Etiology and Pathogenesis of PCOS: Anovulation, Androgen Excess, and Disordered Follicle Development

Oligo- or anovulation is a key clinical feature of PCOS, but there still remains doubt about the mechanism of ovulatory disorders in women with PCOS. The endocrine features of women with anovulatory PCOS include disordered gonadotropin secretion, metabolic abnormalities, and discordant ovarian follicular function. There is much debate about which of these disorders is the primary drive to anovulation, but a strong case can be made for suggesting that there are intrinsic ovarian abnormalities in PCOS that underpin the etiology of anovulation. Anovulation in PCOS is characterized by arrest of antral follicles during the final stages of maturation, that is, typically between 5 and 8 mm in diameter, which is just below that expected of the emerging dominant follicle during a normal cycle.[63] This failure to select, or to complete normal development of, a dominant follicle is almost certainly related to the abnormal endocrine environment. However, there is now evidence that the earliest stages of follicle development, including activation of primordial follicles, are disrupted in anovulatory women with PCOS.

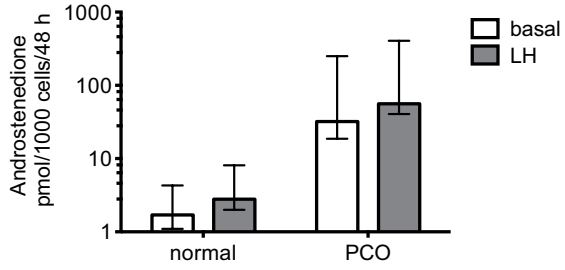

FIGURE 29.10 Production of androstenedione (median, range) by human theca cells in primary culture. Greatly increased androgen production by cells from women with polycystic ovaries is shown. Note the logarithmic scale of the Y-axis. LH stimulated androstenedione production by both normal (1.7-fold) and PCO theca (1.8-fold, $p < 0.005$). *Source: Adapted from Ref. 182.*

Although anovulation is very common in PCOS (and indeed considered to be invariable by the NICHD diagnostic criteria), the biochemical hallmark of PCOS is hypersecretion of ovarian androgen. As described in this chapter in discussing the possible developmental origins of PCOS, ovarian production of excess androgen not only is an important contributory factor in the clinical abnormalities in PCOS, but also may play a central part in the etiology of the syndrome itself. In the remainder of this subsection, abnormalities of antral follicle function (of both theca and granulosa compartments), of related features of gonadotropin secretion, and of preantral follicle development will be described in some detail.

ABNORMAL ANTRAL FOLLICLE DEVELOPMENT: THECA

Hypersecretion of androgens is the most consistent biochemical abnormality in women with PCOS,[181] and the hyperandrogenism is associated with what must be considered to be an intrinsic abnormality of theca cell function. Human theca cells that are derived from women with PCOS have been shown to produce androgens in great excess of the amounts secreted by theca from the ovarian follicles of normal women[182] (Figure 29.10). Production of intermediate steroids in the androgen biosynthetic pathway was also increased, suggesting a global upregulation of steroidogenic enzymes. Such abnormalities of cells in primary culture might be attributed to the abnormal endocrine environment from which the cells were derived (e.g., higher than normal levels of LH and/or insulin), but it is significant that a similar overall increase in steroidogenesis has been noted in theca cells that have undergone several passages in culture.[183,184] Theca cells from polycystic ovaries are

therefore characterized by constitutively increased activity of steroidogenic enzymes. The origin of theca cells in the ovary, and the paracrine signals that guide the recruitment of theca cells to the follicle, remains rather mysterious, and it is legitimate to question whether the primary etiological abnormality in PCOS has its origins in the very earliest stages of follicular development (see the section Abnormal Preantral Follicle Development in PCOS).

ABNORMAL ANTRAL FOLLICLE DEVELOPMENT: GRANULOSA

Perhaps the most important feature in understanding the mechanism of anovulation in women with PCOS is that antral follicles derived from polycystic ovaries show considerable heterogeneity in terms of steroid production and responsiveness to gonadotropins.[185–188] In studies of follicles obtained from unstimulated human ovaries, granulosa cells from small to medium-sized antral follicles (1–9 mm in diameter) taken from women with ovulatory cycles and normal ovaries showed, as expected, a variable response to FSH and no response to LH in terms of production of estradiol and progesterone.[187] There was much more heterogeneity of responsiveness amongst follicles from anovulatory women with PCOS than in follicles obtained from women with a history of ovulatory cycles. Importantly, among small antral follicles (2–9 mm), some were inappropriately responsive to LH.[187] Such premature responsiveness of granulosa cells is likely to result in terminal differentiation of granulosa cells[189] in that subpopulation of follicles and therefore premature arrest of follicle growth. These prematurely advanced follicles are very likely to contribute to circulating estradiol levels in anovulatory women with PCOS that typically are slightly higher than those seen in the early follicular phase of a normal ovulatory cycle. The consequence of this would be suppression of FSH serum concentrations that, whilst in the normal midfollicular phase range, are at a level that is below that seen during the intercycle rise in FSH and consequently below the "threshold" required for the recruitment and progression of antral follicles during the follicular phase. In this way, the further development of normal healthy follicles within this heterogeneous cohort will also be arrested[63,73] (see Figure 29.7). This phenomenon would explain why modest elevation of serum FSH, by the use of antiestrogens or by giving exogenous FSH, usually restores ovulatory cycles; the prematurely advanced follicles remain arrested, but the healthy follicles are able to progress.

As mentioned here, it is the abnormal endocrine environment that seems most likely to be implicated in arrest of antral follicle development. It has been suggested that both hyperinsulinemia and hypersecretion of LH contribute to arrested follicle growth.[77,190,191] Insulin augments the steroidogenic response to LH in cultured granulosa cells from human ovarian follicles.[77] There is evidence that even in a state of peripheral insulin resistance, granulosa cell steroidogenesis remains sensitive to insulin.[77,192]

ABERRANT GONADOTROPIN SECRETION IN PCOS

Abnormalities of gonadotropin secretion are common but not invariable in women with anovulatory or oligo-ovulatory PCOS, and it remains a matter of debate whether such abnormalities are key to the etiology of anovulation or whether they occur secondary to disordered ovarian function. In any case, it is clear that, once established, the pattern of abnormal ovarian function can be sustained by an interaction of hypothalamic–pituitary and ovarian effects. The characteristic pattern is of hypersecretion of LH but with serum FSH concentrations that are typically within the normal midfollicular phase range but are inappropriately low (as discussed in this chapter).[193,194] The pulsatile pattern of LH is often abnormal in that both the amplitude and frequency of LH pulses are increased compared with the profile in the normal follicular phase. However, during a spontaneous or induced ovulatory cycle, the pattern of LH secretion normalizes, indicating that aberrant gonadotropin patterns are due, at least in part, to inappropriate negative feedback (i.e., low estradiol, particularly lack of cyclical progesterone).[194] Nevertheless, abnormal LH secretion, if less obvious, may still be observed even in women with polycystic ovaries who have ovulatory cycles,[195] together with evidence of impaired progesterone-mediated suppression of LH. Women with polycystic ovaries had serum LH levels that were significantly less suppressed during treatment with a combined oral contraceptive than in control subjects with normal ovaries[196] (Figure 29.11). This phenomenon may be attributable to programming of the hypothalamic–pituitary axis by exposure to excess ovarian androgen during development rather than a primary hypothalamic–pituitary

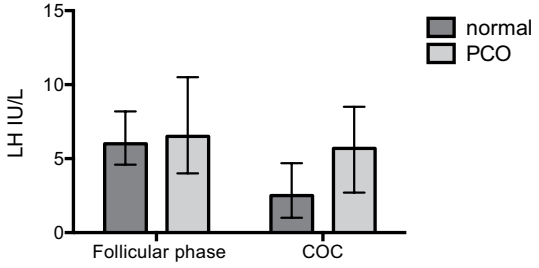

FIGURE 29.11 Serum LH concentrations in women with polycystic ovaries on ultrasound who were studied in the follicular phase of spontaneous cycles or whilst taking the combined oral contraceptive (COC). Note the reduced suppression of LH ($p < 0.05$) in women with PCOS during COC treatment. *Source: Data adapted from a study of the prevalence of PCOS in women in the general population by Ref. 196.*

defect. There is strong evidence to support this from studies of prenatally androgenized monkeys or sheep and also from clinical studies. Girls with adrenal hyperandrogenism associated with congenital adrenal hyperplasia may have elevated LH serum levels,[197] and, in an elegant series of studies, Marshall and colleagues demonstrated that impaired progesterone-mediated negative feedback suppression of LH in women with PCOS could be reversed by treatment with an androgen receptor antagonist.[198,199] These findings support the notions that the primary defect in PCOS resides within the ovary and that changes in the regulation of gonadotropin secretion are consequent upon abnormal ovarian steroidogenesis. Further and direct evidence for an intrinsic and primary ovarian abnormality comes from the studies of the early stages of follicle development (i.e., at a stage when local ovarian factors appear to be more important than gonadotropins in directing the activation and early growth of preantral follicles).[63]

ABNORMAL PREANTRAL FOLLICLE DEVELOPMENT IN PCOS

As summarized in the section Preantral Follicle Development, the time taken from the activation of primordial follicles through their growth to the late preantral stage in the human ovary is estimated to be 3–6 months.[52,53] However, we know little about the factors involved in regulation of normal preantral follicle development and even less about what dictates the aberrations seen in PCOS. Nevertheless, there are characteristic morphological and functional differences between normal and polycystic ovaries in these earliest stages of follicle development. There is a greater density of preantral follicles in the polycystic ovary.[64] The increased density of preantral follicles appears to be largely due to an increase in the primary follicle "pool",[200,201] a phenomenon referred to as "stockpiling" of follicles by Maciel and colleagues.[201] Furthermore, studies of cultured cortical tissue biopsies revealed that the proportion of follicles that initiated growth was significantly higher, and the proportion of primordial follicles reciprocally lower, in tissue from women with polycystic ovaries than in biopsies from normal ovaries. In other words, the dynamics of early follicle development are altered in women with PCOS. Despite the evidence for accelerated activation of primordial follicles, there is nothing to suggest that this leads to premature depletion of the follicle pool. The age of the menopause appears to be no different in women with proven PCOS from those in a reference population.[152] Preservation of the follicle pool in PCOS may be attributable to a higher starting population of preantral follicles,[200] reduced atresia of preantral follicles,[202,203] and/or the accumulation (stockpiling) of follicles at the primary stage.[201]The underlying reasons for the increase in preantral density in polycystic ovaries remain obscure, but these include, plausibly, increased endowment of primordial follicles and reduced loss of follicles by atresia during the early stages of growth (Figure 29.12). Clearly, it is difficult to find direct evidence for the former, but data from tissue culture studies support the notion that there is reduced atresia of preantral follicles.[202]

Accelerated initiation of growth in polycystic ovaries is associated with a higher than normal proportion of granulosa cells and evidence of a higher rate of cell division within the granulosa layer of primordial and early growing follicles. Expression of mini-chromosome maintenance protein2 (MCM2, a marker of DNA replication)[204–206] was increased in follicles from PCOS patients.[207] The observation that MCM2-positive granulosa cells were present even in primordial follicles

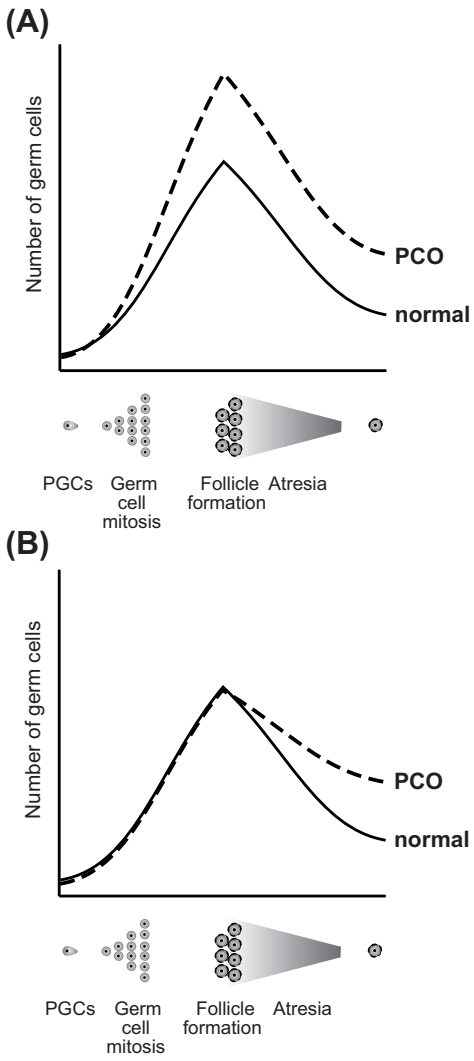

FIGURE 29.12 Proposed mechanisms for increased density of preantral follicles in women with PCOS. (A) Possible mechanisms for increased initial density are (i) more cell division in PGCs leading to (ii) increased endowment of primordial; and/or (B) increased survival of preantral follicles. *Source: Adapted from Ref. 200.*

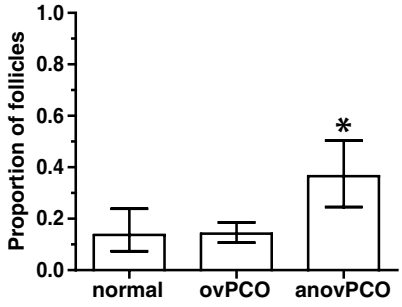

FIGURE 29.13 Granulosa cell proliferation is increased in preantral follicles of women with PCOS. Proportion of preantral ovarian follicles expressing the proliferation marker mini-chromosome maintenance protein2 (MCM2) in women with normal ovaries, and two groups of women with PCOS—those with regular ovulatory cycles (ovPCO) and those with anovulation (anovPCO) (*$p = 0.03$). *Source: Data from Ref. 207.*

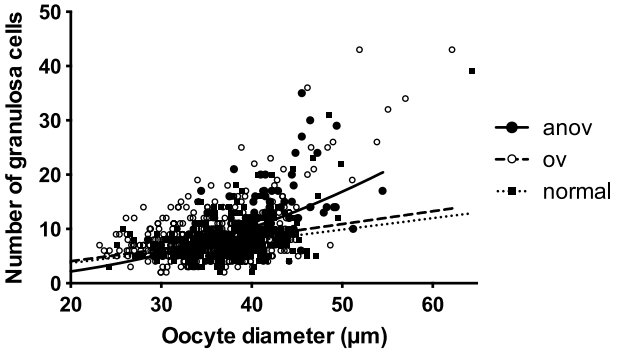

FIGURE 29.14 Relationship between granulosa cell number and oocyte diameter in preantral follicles from normal and polycystic ovaries. Note the discordant pattern in follicles from women with anovulation and PCOS.[207]

suggests that, contrary to current dogma, these follicles are not completely quiescent and there may be a low level of cell division even in this "resting" reserve of follicles. Preantral follicle development, from primordial up to and including the primary stage, was marked by a higher proportion of MCM2-positive follicles in tissue from anovulatory women with polycystic ovaries than in tissue from either of the two ovulatory groups (Figure 29.13). In addition, it was observed that oocytes were larger and there were more granulosa cells per follicle in transitional and primary follicles in polycystic ovaries from an anovulatory PCOS group. However, the increase in the number of granulosa cells was disproportionately greater than the increase in oocyte diameter in the follicles, emphasizing the altered dynamics of early follicle development in polycystic ovaries and implying an alteration in the normal "dialog" between oocyte and somatic cells (Figure 29.14).

It is not clear, as yet, which (presumably intraovarian) factors are involved in abnormal regulation of early folliculogenesis, but both IGFs[76,208–210] and androgens have been implicated, mainly on the basis of studies in nonhuman animal models. In the rhesus monkey, ovarian androgens have been shown to stimulate initiation of follicle development, and this effect may be mediated by increased expression of the type 1 IGF receptor.[209,210] Aberrant follicle development has been observed in the ovaries of prenatally androgenized rhesus monkey and sheep.[166,211–214] If these data are to be extrapolated to the human, they beg the question as to the source of excess androgen. It is most likely to be produced by theca cells of the ovary, but since theca formation is a key element of normal follicle development, it is unclear whether excess production of androgen by theca (an intrinsic abnormality in the polycystic ovary, as discussed in this chapter) is the primary abnormality or whether it is, itself, a consequence of abnormal preantral follicle development. Nevertheless, it seems plausible that, once established, excess androgen production by theca cells contributes to the perpetuation of abnormal dynamics of early follicle development.

Another key question is whether these abnormalities in preantral follicle development are related to the disordered antral follicle function described in this chapter (and, if so, by what mechanism is this brought about?). The argument has been made for arrest of antral follicles being a reflection of the abnormal endocrine environment in anovulatory women with PCOS, but might the disorder of early follicle growth predispose the follicle to an inappropriate responsiveness to endocrine signals? For example, is the premature response to LH of granulosa cells in small antral follicles simply a function of the endocrine environment, or do these cells also respond differently to LH because of their early programming?

INSULIN RESISTANCE IN PCOS

The idea that insulin resistance was a feature of women with PCOS was mooted in the 1970s, but the first clear evidence was in 1983 when Chang and colleagues noted an exaggerated insulin response to an oral glucose load in a group of women with PCOS when compared with weight-matched controls.[215] This was followed by the seminal work of Dunaif and coworkers who used hyperinsulinemic–euglycemic clamp studies to assess directly insulin sensitivity in groups of hyperandrogenemic women.[148,216,217] Insulin resistance is most obvious in overweight and obese women with PCOS but can also be observed in lean women with the syndrome, particularly in carefully weight-matched groups[217,218] (Figure 29.15). More recently, and following the debate about diagnostic criteria for PCOS, it has become clear that insulin resistance is a feature of women with PCOS who have both hyperandrogenism and anovulation, whereas those with polycystic ovaries who have either hyperandrogenism or anovulation alone tend to have normal insulin sensitivity.[219–221] However, it should be noted that recent data from hyperinsulinemic–euglycemic "clamp" studies

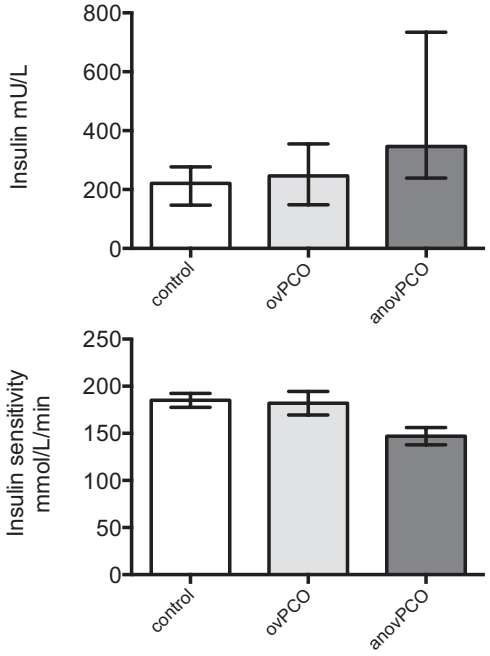

FIGURE 29.15 Sum of serum insulin concentrations during a standard 75 gm oral glucose tolerance test (median and interquartile range), and insulin sensitivity (mean ± SEM) in three groups of weight-matched subjects: women with normal ovaries and regular cycles; women with polycystic ovaries, androgen excess, and regular cycles (ovPCO); and women androgen excess and anovulation (anovPCO). Hyperinsulinemia and insulin resistance are features of anovPCO but not ovPCO. *Source: Data from Ref. 190.*

suggest that these intermediate groups may be mildly insulin resistant relative to controls.[222] But even within the group with both features of PCOS, there is a wide range of insulin sensitivity, with considerable overlap of values with the normal range. In other words, insulin resistance is a common but not invariable feature of women with PCOS, even in overweight or obese subjects.

Insulin resistance in PCOS has been linked to a disorder of energy balance characterized by reduced postprandial thermogenesis (PPT).[218] PPT accounts for about 15% of daily energy expenditure, and reduced PPT may represent a mechanism by which women with PCOS have an increased capacity to gain weight. This brings to mind the concept of the thrifty genotype hypothesis wherein such a "defect" in energy expenditure can represent a biological advantage in terms of survival and, particularly, preservation of reproductive performance (the thrifty reproductive genotype[223]) at times of food shortage. However, it remains debatable whether this quite subtle disorder of energy balance is enough to account for a tendency to gain weight readily in women with PCOS. In fact, obesity is only slightly more common amongst women with symptoms of PCOS in the general population than in those without PCOS.[224] But, of course, weight gain is associated with more symptoms of PCOS and therefore a greater likelihood of presenting to a clinic.

TABLE 29.6 Summary of the Key Investigations in Women with Anovulation

- Serum FSH and LH
- Serum prolactin
- Assessment of estrogen production (serum estradiol and endometrial thickness)
- Serum TSH

The cellular and molecular mechanisms of insulin resistance in PCOS seem to have characteristics that are peculiar to the syndrome. As in many types of insulin resistance, there is evidence for a postreceptor defect and pathway-specific defects in insulin signaling. Dunaif has proposed that there is an increase in serine phosphorylation of both the insulin receptor and insulin receptor substrate 1 that affects metabolic but not mitogenic pathways, not only in classic insulin target tissues but also in the ovary. Abnormalities of serine phosphorylation within the mitogen-activated protein kinase (MAPK)–ERK signaling pathway have also been noted.[146] Selective insulin resistance in the ovary—affecting metabolic but not steroidogenic function—has been alluded to previously. More specifically, insulin-mediated glucose uptake and lactate accumulation have been shown to be impaired in granulosa–lutein cells of women with PCOS.[192,225,226] It is possible that this defect contributes to the subfertility of PCOS by affecting the energy supply of the oocyte, but there is no direct evidence for this. Indeed, oocyte quality appears to be little different from normal in women with PCOS.[227] Insulin-sensitizing drugs can improve the chance of ovulation in women with PCOS,[161] but this is probably effected by lowering the hyperinsulinemic drive to excess steroidogenesis.

Clinical Management of Ovulatory Disorders

Investigation of Ovulatory Disorders

In clinical practice, investigation of patients with ovulatory disorders requires only a small number of well-directed tests[228] (Table 29.6). In patients presenting with amenorrhea, measurement of gonadotropins and prolactin and assessment of estrogen production will provide the essential information, and usually a single blood sample is all that is required. Measurement of FSH will immediately allow distinction between POI and other causes of oligo-amenorrhea, and, in women with normal (or frankly low) FSH, assessment of estrogen status will discriminate between hypothalamic–pituitary causes (low estrogens) and PCOS (normal estrogen status). Although estradiol measurements are routine in most reproductive endocrine clinics, there is considerable overlap between physiological levels of estradiol in the early follicular phase of normally cycling women and

values seen in women with estrogen-deficient amenorrhea. It may be advantageous, therefore, to evaluate estrogen production, using an "in vivo bioassay" of estrogen action by measuring endometrial thickness of ultrasound in patients presenting with amenorrhea. Additionally, in women with low or normal FSH, measurement of prolactin will identify those women who have hyperprolactinemia as the cause of the hypothalamic amenorrhea.

The requirement for additional investigations depends primarily on the clinical presentation but also on the results of the initial tests described here. So, for example, it is important to perform pituitary imaging in women with hyperprolactinemia or serum testosterone in hirsute women with symptoms of PCOS. The approach to any further investigation and treatment of the individual disorders is described throughout the remainder of this section.

Treatment of Ovulatory Disorders

The main focus of the treatment of women with ovulatory disorders will be on restoration of normal ovulatory function (i.e., predominantly in the treatment of infertility). However, management of patients with amenorrhea or oligomenorrhea also includes, in women not wishing to conceive, treatment of associated symptoms and long-term consequences of hormone deficiency, as well as treatment of the effects of hormone excess (e.g., unwanted body hair due to hyperandrogenism).

The results of the few simple tests outlined here inform the optimal choice of treatment for induction of ovulation. With the exception of women with POI (in whom fertility can realistically be restored only by egg donation, with the help of in vitro fertilization (IVF)), disorders of ovulation are eminently treatable. A very important objective in induction of ovulation is to restore normal ovulatory cycles (i.e., to aim for single-follicle ovulation). This is in marked contrast to superovulation (often euphemistically called "controlled ovarian stimulation") in the context of an IVF program where the objective is to override physiology and recover several eggs for IVF.

PRIMARY OVARIAN INSUFFICIENCY

Different fertility options will be appropriate for each individual with POI. It is important that discussions on this topic, from a professional with experience in the field, are made available not only soon after diagnosis but also at intervals throughout follow-up as the technology and opportunities in this field are continually developing. Women with POI have a 5–10% chance of spontaneous conception at some time after diagnosis, as in some cases hormone levels and disease activity fluctuate and return to biochemical normality; this is often transient, and the likelihood of recovery of ovulation is not possible to predict. Pregnancy loss in those who conceive is reported at 20%, which is similar to that of the normal population. Several medical therapies have been tried to induce ovulation in women with POI; however, in a systematic review all were reported to be equally ineffective.[87] In particular, glucocorticoids have been considered for those with autoimmune markers, and although they were promising in early case reports, a controlled trial failed to show benefit.[87] With fluctuating ovarian function being more common in POI than is often realized, it may be tempting to offer close ovarian monitoring to detect times of remission.[229] This technique has yet to be shown to be a significant advance and runs the risk of raising false hope in many to achieve pregnancy in a few.

OVUM DONATION

Assisted conception with donated oocytes has been used to achieve pregnancy in women with POI for over 20 years and remains the only realistic fertility treatment for the majority of women with established POI.[230,231] The availability of donated oocytes varies from country to country, and this option is not acceptable from some ethnic groups. Donated oocytes are fertilized with partner's sperm and the best embryo is chosen for implantation with the recipient, having been prepared with increasing doses of oral estradiol. Because of the lack of a corpus luteum, progesterone support is required that can be administered orally, vaginally, or by injection. Endometrial preparation is established in earlier "dummy" cycles in which endometrial thickness can be tracked by ultrasonography without embryo transfer.[232]

EMBRYO CRYOPRESERVATION

Cryopreservation of embryos is a long-established technique as part of IVF, but it will apply to only a small number of women with normal ovarian function who can anticipate imminent ovarian failure but have sufficient time to allow for ovarian hyperstimulation.[233] This circumstance might arise in some cases of malignancy or in someone who is known to be at risk of ovarian failure, such as those with X chromosome anomalies or FMR1 premutation who may wish to delay starting a family for some years. Donor sperm could be used for those without a partner.

OOCYTE CRYOPRESERVATION

Cryopreservation of oocytes has only been a practical option since the advent of vitrification, which has improved the freezing process for such a large cell.[234] Vitrification involves rapid cooling in high concentrations of penetrating cryoprotectants, which avoids formation of intracellular ice and resulting damage during cooling and warming. In vitro maturation of oocytes, where immature oocytes are retrieved from unstimulated

ovaries, has also emerged as a safe and effective treatment for women with cancer who are undergoing gonadotoxic therapy. Successful pregnancy is possible following in vitro maturation of oocytes from antral follicles, but this is not yet established in clinical practice.[235]

Oocyte cryopreservation also requires controlled ovarian stimulation, but in contrast to preservation of embryos, no sperm is required. In practice, this option is only applicable to women with normal ovarian function and beyond early adolescence. The success rate for achieving pregnancy is likely to be lower than for embryo cryopreservation because oocyte survival and subsequent fertilization are impaired. This option has been used for those women with Turner syndrome who retain some ovarian function,[236] but so far the overall success rate is not known for this subgroup compared to the more common application in women diagnosed with cancer.

OVARIAN TISSUE CRYOPRESERVATION

The use of ovarian tissue cryopreservation for later use has been explored in young women undergoing anticancer treatment. Cryopreservation of one ovary or strips of ovarian cortical tissue has resulted in the birth of over 12 children according to a review in 2012.[237] This procedure has the advantage of not requiring sperm and being appropriate for prepubescent girls accepting that a laparoscopy is required. The cryopreserved tissue can be reimplanted into the pelvis, with ovulation and pregnancy occurring spontaneously or after IVF. Once again, it is too early to be certain of the overall success rate of this procedure, which is only undertaken in specialist oncology centers. In rare instances, successful pregnancy following ovarian transplantation between monozygotic twins discordant for ovarian failure is possible, with 28 births reported.[238]

Disordered Regulation or Deficiency of Gonadotropins

Gonadotropin deficiency caused by destructive lesions of the pituitary or hypothalamus, although rare, will usually be obvious in the context of the clinical presentation, and the need for additional tests (particularly assessment of other anterior or posterior pituitary hormones and detailed imaging) can be considered. With increasing knowledge about *genetic causes of isolated GnRH deficiency*, it is appropriate to screen for known genetic mutations implicated in hypothalamic amenorrhea, particularly when there is a relevant family history.

In women with FHA, treatment of the underlying cause is the priority. It is usually obvious when an eating disorder is the predominant cause of FHA, but in those who do have weight loss-related or exercise-related amenorrhea, it is not simply a matter of advising that they eat more healthily or exercise less vigorously; psychological support is very important in management.[129] Even in those with idiopathic FHA, psychotherapy in the form of cognitive-behavioral therapy may be indicated and has been shown to be effective.[239]

PULSATILE GNRH TREATMENT

The most physiological approach to induction of ovulation is pulsatile GnRH. This is the treatment of first choice in women with organic causes of GnRH deficiency and in those women with FHA who have regained weight, reduced exercise, and/or benefited from psychological therapies but do not resume menses. The advantage of GnRH therapy over exogenous gonadotropins is that the pituitary–ovarian feedback is maintained, restricting the pituitary secretion of FSH in the follicular phase, encouraging the development of a single dominant follicle, and reducing the risk of multiple-follicle development. By contrast, it is not easy to find the appropriate threshold dose of exogenous FSH at which a single dominant follicle is selected, and consequently there is a significant risk of exceeding this threshold and inducing multiple-follicle development. Since the initial "proof-of-principle" studies of the efficacy of pulsed GnRH cited in this chapter, there have been significant refinements with regard to dose and route of administration that have led to effective and convenient management of women with hypogonadotropic hypogonadism.

The initial clinical studies with the use of pulsatile GnRH were performed in the late 1970s and early 1980s, with groups based in Bonn, Boston, Uppsala, and London being the most prominent in the field.[38–40,240,241] Homburg and colleagues reported a cumulative conception rate of 93% in a series of 118 women, 61 of whom had disordered regulation or deficiency of gonadotropins. The prevalence of multiple pregnancy (twins) was, as predicted, lower than that expected after gonadotropin therapy, at 7%. The results from the Boston series were equally impressive across a wide range of causes of GnRH deficiency. Interestingly, subsequent studies have suggested that even patients with mutations in the GnRH receptor may be amenable to treatment with pulsed GnRH, albeit at larger doses.[242] The same group has recently reported a relationship between response to GnRH and genotype in women with isolated GnRH deficiency.[57] Mutations in *CHD7*, *FGFR1*, *KAL1*, *TAC3*, and *TACR3* were associated with a favorable response to GnRH, whereas those women with mutations in *GNRHR* and the prokineticin receptor 2 gene (*PROKR2*) demonstrated pituitary resistance to GnRH therapy. The poor response to GnRH was predictable in patients carrying mutations in the GnRH receptor gene but is less easy to explain in those with a mutation in *PROKR2* (although the latter has been implicated in normal pituitary development). This emphasizes the emerging importance of genetic screening of such patients in the selection of appropriate treatment.

GONADOTROPIN THERAPY

Modern delivery systems (automatic pulsatile infusion pumps) for administration of GnRH are small and unobtrusive but nevertheless need to be worn daily until pregnancy is achieved. This may be a problem for some women, and an alternative is to give exogenous gonadotropins by daily injection. In contrast to the gonadotropin requirements for induction of ovulation in PCOS (or for superovulation for IVF), administration of FSH alone is not sufficient to ensure normal follicular maturation. Women with hypothalamic amenorrhea lack LH and therefore cannot generate enough androgen substrate for production of estradiol. Thus, a combination of LH with FSH is needed, most conveniently in the form of human menopausal gonadotropin (hMG). But because gonadotropin therapy carries a much greater risk of hyperstimulation of the ovaries than does GnRH, it demands more intensive monitoring. For this reason, pulsatile GnRH remains the treatment of first choice for women with hypothalamic amenorrhea. In view of the results of GnRH treatment in women with primary pituitary disease, mentioned in this chapter, a trial of pulsed GnRH may be appropriate even in those women with GnRH receptor mutations or destructive pituitary lesions. Nevertheless, this is a group in whom exogenous gonadotropin therapy is more likely to be effective. Of course, this introduces the possibility of overstimulating the ovaries, but there is limited evidence to suggest that the risk of multiple-follicle development in response to gonadotropins can be reduced by the addition of, or replacement of FSH by, LH in the final stages of follicle maturation.[243,244]

PROSPECTS FOR FUTURE MANAGEMENT

The rapidly emerging knowledge of the GnRH pulse generator and of the endocrine factors that impact it raise the prospect of new methods of restoring pulsatile secretion of GnRH for fertility treatment. A good example of this is the recent work on administration of kisspeptin to women (and men) with GnRH deficiency. In preliminary studies in males, constant infusion of kisspeptin has been shown to induce episodic LH secretion,[245] although it is not clear that the pulse pattern is normal, nor is it yet certain that this will restore normal cyclical ovarian function in women. Much more needs to be learned regarding many issues, including dose-response effects, selection of patients for clinical trials, and how to create effective long-acting analogs for routine clinical use. Another exciting area in the field of endocrine therapies for anovulatory infertility is the development of small molecule mimetics of gonadotropins for oral use. These allosteric modulators of gonadotropin receptors have potential for both pro-fertility and contraceptive application.[246]

Hyperprolactinemia

After excluding pharmacological causes of hyperprolactinemia and primary hypothyroidism, the focus of treatment is direct inhibition of prolactin secretion using long-acting dopamine agonists.[32,247,248] Although hyperprolactinemic amenorrhea is characterized by hypothalamic dysfunction and abnormal GnRH pulsatility, it is much more appropriate to use dopamine agonists (the most commonly used are bromocriptine and the longer acting cabergoline[249]) to inhibit prolactin secretion than to give pulsatile GnRH. Suppression of prolactin to within or near the normal range results in resumption of normal pulsatile secretion of gonadotropins and return of ovulatory menses.[32] Importantly, dopamine agonists also reduce the mass of prolactin-secreting pituitary adenomas, not only in the case of microadenomas but also in many patients with large prolactinomas. It is therefore only rarely necessary, at least in the short term, to perform pituitary surgery, even in women who have large enough tumors to cause local symptoms such as visual field loss. In the long term, microadenomas may apparently be cured by dopamine agonist therapy, but the rate of recurrence for microadenomas is much higher and, although long-term dopamine agonist treatment carries very few risks, surgical treatment may eventually be required.[250] In a small proportion of patients, dopamine agonist treatment is not well tolerated and has to be discontinued because of side effects. Postural hypotension, nausea, and nasal stuffiness are common but usually resolve if the starting dose of bromocriptine or cabergoline is low and then gradually increased to a level that provides effective suppression of prolactin. Resistance to dopamine agonist therapy is uncommon but well recognized.[251]

Polycystic Ovary Syndrome

DIET AND LIFESTYLE MODIFICATION

Overweight and obesity in women with PCOS are associated with both a reduced frequency of ovulation and a poorer response to ovulation induction. Lifestyle modification, specifically calorie restriction and structured exercise programs leading to modest weight reduction of between 5% and 10%, has been shown not only to improve metabolic indices in women with PCOS but also to increase the chances of ovulation and pregnancy.[158–160] For the most part, these studies, although consistent in their findings, have been on a relatively small scale, and we lack systematic data both on longer term outcome and on whether overweight or obese women who respond poorly to induction of

ovulation will show an improved response following weight loss.

ANTIESTROGENS

The treatment of first choice for induction of ovulation in anovulatory women with PCOS is the antiestrogen clomiphene citrate, which results in ovulation in 75–80% of patients.[252,253] The antiestrogenic effect of clomiphene at the level of the pituitary and hypothalamus results in the removal of estrogen-mediated negative feedback and a significant increase in serum FSH, sufficient in most cases to exceed the threshold for further development of medium-sized antral follicles. The usual starting dose is 50 mg per day for 5 days (from day 2 onward) following a spontaneous period or progestin-induced withdrawal bleed. It is prudent to monitor the first cycle of treatment by ultrasound tracking and endocrine tests in order to determine whether ovulation has occurred or whether there has been either a poor response (no dominant follicle emerging) or an excessive response (too many follicles). The dose can then be adjusted accordingly, but there is no clear evidence that increasing the dose above 150 mg per day can improve the ovulation and pregnancy rate.[253] There are promising data to suggest that a useful alternative to antiestrogens is the use of aromatase inhibitors. These have been shown effectively to induce ovulation in women with PCOS. These agents also induce a rise in serum FSH by removal of estrogen-negative feedback, but, in this case, this is due to a transient fall in circulating estradiol concentrations rather than estrogen receptor blockade. They appear to have fewer side effects than clomiphene, but progress in bringing aromatase inhibitors into fertility treatment has been slowed following concerns about possible teratogenicity, albeit in data arising from small, preliminary studies. At the time of writing, a large, multicenter, randomized controlled trial is in progress with the focus on safety for the offspring as well as on efficacy.[254]

GONADOTROPIN THERAPY AND LAPAROSCOPIC OVARIAN DIATHERMY

In women who fail to ovulate on clomiphene (or in those who ovulate but have not conceived after successive ovulatory cycles), the choice of treatment lies between laparoscopic ovarian diathermy (although just how this works remains obscure)[255] or low-dose FSH[253] with careful ultrasonographic monitoring of the ovarian response.[256] Both treatments have proven effective in treatment of clomiphene nonresponders, and the choice is usually determined by a combination of patient preference and availability of specialist resources. The advantage of laparoscopic diathermy (the modern replacement for the time-honored treatment of ovarian wedge resection) is that it is a single-step procedure and it may be particularly appropriate in women with PCOS who are due to undergo routine laparoscopy as a diagnostic procedure during infertility investigations. The disadvantages are that it is an invasive procedure, that adjuvant treatment with clomiphene or gonadotropins is required in about half of the cases, and that it is less effective in obese than in lean women with PCOS. Gonadotropin treatment is equally, if not more, effective, but women with PCOS are more prone to multiple-follicle development and the attendant risks of multiple pregnancy and ovarian hyperstimulation syndrome (OHSS). These undesirable, and dangerous, complications of gonadotropin treatment have led to the development of low-dose, "step-up" gonadotropin treatment regimens.[147,249,256,257] These protocols are based on the pioneering work of Brown and colleagues[67] who argued that by carefully increasing the dose of gonadotropin by small amounts (in order to find the appropriate "threshold" level of FSH for a single dominant follicle to emerge), there was a much greater likelihood of allowing single-follicle ovulation. In our own series of nearly 200 women treated with low-dose FSH, the rate of single-follicle ovulation was over 80% and the incidence of both multiple birth and OHSS was very low (Table 29.7).[258] The key feature of this treatment regimen is that the dose of FSH is sufficiently low to simply "top up" endogenous levels of FSH to the range normally seen in the early follicular phase of the cycle. A glance at the endocrinology of a treated follicular phase reveals that even if the threshold dose is "clamped", the endogenous concentrations of FSH fall in the late-follicular phase, as in the normal cycle. In other words, the normal physiological negative feedback (by increasing levels of estradiol) is maintained with this regimen. It will also be noted that LH levels, which are elevated at the beginning of the treatment cycle, are suppressed into the normal range in the mid- to late follicular phase of the cycle (Figure 29.16). This low-dose, step-up regimen is now widely accepted as the treatment of choice for gonadotropin-induced ovulation in women with PCOS.[147]

TABLE 29.7 Outcome of Treatment with Low-Dose FSH in 199 Women

Cycles	916	
Ovulatory cycles	657	72%
Uniovulatory cycles	562	86%
Pregnancies	91	46%
Miscarriages	21	23%
Multiples (all twin)	3	3%

Mild "OHSS" in 4% of cycles.
From Ref. 258.

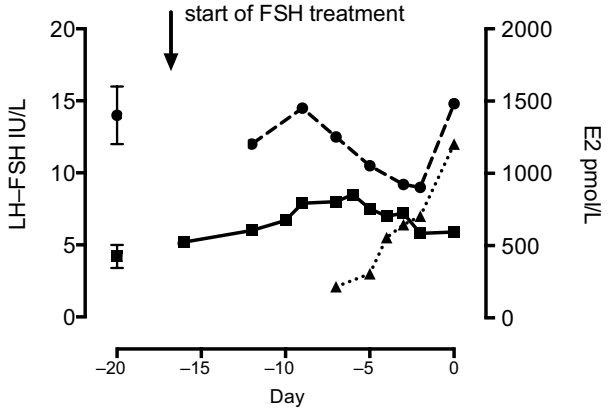

FIGURE 29.16 Mean serum LH (filled circles, day −20) and FSH (filled squares, day −20) concentrations before treatment; and LH (filled circles with dashed lines), FSH (filled squares with solid line), and estradiol (filled triangles with dotted line) during induced mono-ovulatory cycles in women with anovulatory PCOS treated with low-dose FSH. Serum FSH levels are raised to the normal early follicular phase range but show a fall toward day 0 (days normalized to start of the LH surge or injection with human chorionic gonadotropin), indicating preservation of the normal negative feedback control of FSH in the late follicular phase despite a fixed dose of exogenous FSH from day 10 onward. Note also the suppression of high LH levels during the late follicular phase.

CONCLUSION

Our understanding of the physiology of human ovarian function and its control has increased enormously in the last 50 years, aided in no small part by the widespread application of radioimmunoassay to elucidate not only normal cyclical changes in gonadotropins and sex steroids but also the etiology of disorders of ovulation. In the last decade, this knowledge has been greatly enhanced by huge advances in the fields of genetics and cell signaling, but our understanding of the physiology of the ovarian cycle remains the bedrock for investigation and successful management of ovulatory dysfunction in women.

Disorders of ovulation are a very common cause of female infertility, and although the etiology of, and the principles of management of, the various causes of anovulation are now well understood, there remain many areas of uncertainty. These uncertainties are the basis for further research into the physiology and pathophysiology of female reproduction.

In the field of POI, the issue of whether there are stem cells (oogonial stem cells) in the germ cell line within the adult human ovary is an intriguing but highly controversial area that merits further research. If it is indeed possible to generate new oocytes from such stem cells, this has the potential to offer prolongation of the reproductive lifespan in women whose ovarian reserve is low. Of more immediate therapeutic application, however, is the ongoing research using cryopreserved fragments of ovarian tissue for restoration of fertility in young women who are about to receive chemotherapy or pelvic irradiation for cancer.

In women with hypogonadotropic hypogonadism, the role of genetic factors in the etiology of abnormalities of GnRH secretion is assuming increasing importance. Even in those with FHA in whom environmental influences are paramount, there is evidence that there may be a genetic predisposition to disordered regulation of gonadotropins. Continuing research into afferent neural pathways involved in regulation of GnRH networks has important implications for management of hypogonadotropic hypogonadism. For example, the therapeutic potential of kisspeptin and its analogs in restoring normal ovulatory cycles in women with hypogonadotropic hypogonadism is beginning to be explored.

PCOS is perhaps the most complex disorder of reproductive function in women, and it seems that several factors, genetic and dietary in particular, play a role in its etiology. Unlike hypogonadotropic hypogonadism (in which single gene defects are more common), PCOS is a complex endocrine trait in which several susceptibility loci are likely to be implicated, but to date we have insufficient data to be confident about which are key genetic variants. A role for prenatal exposure to androgens in developmental programming of PCOS is an attractive hypothesis with support from studies of both animal models and women with PCOS. However, there is much more that needs to be learned about the mechanisms involved, in particular the putative interaction of genetic, epigenetic, and environmental factors.

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CHAPTER

30

Puberty in Mice and Rats

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INTRODUCTION

In its 125th-year celebration issue published in 2005, *Science* magazine listed the problem of what triggers puberty, the period of postnatal development during which the capacity for sexual reproduction is achieved, as one of the "125 great unanswered scientific questions" of our times.[1,2] In accordance with its continuing fascination as a subject, there has been no dearth of reviews recapitulating the history of research into puberty, from the sweepingly broad[3–5] to those whose viewpoint is more focused on certain species, mechanisms, or even molecules.[6–15] In the latter category, homage must be paid to the previous version of this chapter, written by Ojeda and Skinner, which still serves as a model and an inspiration for all subsequent efforts to tackle the topic of puberty in rodents.[16] Indeed, any undertaking of this sort runs the risk of not being completely original, unless history can be rewritten or previously undiscovered reports brought to light (as does occur sometimes; see the reference to a little-noticed French paper from 1970 by Dolais et al.[17] on pulsatile luteinizing hormone (LH) release in men and women, mentioned in Knobil, 2005).[18]

With that in mind, the history of the study of puberty in rodents, like the study of most other scientific subjects, is a glittering patchwork of hard-to-believe hypotheses; indeed, one could almost say leaps of faith, formulated by intellectual giants who often had nothing more to go on than fairly crude experimental techniques and their own faculties of meticulous observation. The eventual proof of the accuracy of these hypotheses was brought years, sometimes decades, later, by other giants with better technology and perhaps more time or manpower or funding at their disposal. And one should not discount the role of serendipity in all this—chance encounters with the right people at the right time seem to underlie many of the most important scientific and technical insights leading to the demystification of the process of puberty and, indeed, of sexual differentiation and function, from which it is inseparable.

Although the earliest conjectures concerning the process of sexual maturation and its functional components were based, as could be expected, on observations of human patients and domesticated animals, by the beginning of the twentieth century, experiments in rodents were already yielding significant insights. At the gonadal level, for example, Marshall and Jolly described the effects of spaying pregnant rats as early as 1905,[19] and used these results as well as others to postulate that the ovary released two distinct hormones, later called oestrin/estrin (or estrogen) and progestin (or progesterone), which had different functions (recapitulated in Marshall's Croonian Lecture, 1936[20]). Perhaps with even greater foresight, Steinach in 1910 suggested that sexual development and behavior in rats was controlled by the actions of the gonads on the brain, and that ovarian hormones were related to testicular ones (Ref. 21; retold in Ref. 22). Among the first studies to directly address the question of puberty in rodents was that of Hermann and Stein, who found in 1916 that injecting ovarian and placental "lipoids" delayed puberty in immature male rats.[23] The classic example, of course, is the work of Long and Evans in 1922, whose experiments revealed that the ovaries of immature female rats (of their now famous Long–Evans strain obtained by crossing wild Norwegian grays with Wistar albinos) could be induced to undergo maturation when transplanted into adult females.[24] This was followed in 1924 by the work of Allen and Doisy[25] showing that an ovarian follicular extract was capable of inducing vaginal opening in immature female rats, the first direct experimental evidence for the existence of estrogen, which Marshall had postulated two decades earlier. This discovery became the subsequent basis of the Allen–Doisy test, in which a substance suspected of containing estrogens is injected into an ovariectomized mouse, and vaginal smears examined for the presence

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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of cornified epithelial cells (changes in vaginal cytology during the estrous cycle having already been described by Stockard and Papanicolaou[26] in guinea pigs in 1917). Using the same assay, in 1925, Frank and Gustavson, and later in the year, Frank, Kingery, and Gustavson,[27,28] while investigating whether the normal waiting period to puberty in Long and Evans' rats was due to "restraining influences…at work" or due to the "absence of the female sex hormone", postulated that lipoid extracts of ovarian follicles, corpora lutea, and the placenta, which presumably all contained the same hormone, were capable of inducing vaginal opening and hastening the first ovulation in immature rats. In other words, the time to puberty, in females at least, was not determined by the presence of an inhibitory factor in immature animals, but by the time it took for their ovaries to produce estrogen. In immature males, however, the injection of relatively pure estrin delayed the appearance of primary and secondary sexual characteristics and reduced testicular development in rats and guinea pigs.[29]

While these important discoveries regarding the secretion of ovarian hormones and their influence on puberty, both in males and females, were taking place, other studies were already confirming that the anterior pituitary (also called the pars distalis for its location, and the adenohypophysis or pars glandularis for its hormone-secreting abilities) and not the ovary determined the unfolding of the process. For example, Parkes in 1929 showed that the ablation of the ovarian follicles before puberty, or even in utero in fetal mice, prevented ovarian cycling, but did not affect other changes linked to sexual maturation (Ref. 30; see Ref. 31 for a more detailed account of early experiments on the hypophysial–ovarian relationship). Among their other findings, Long and Evans in 1922 had also shown that the injection of pituitary extracts into female rats would induce ovarian follicles to be converted into luteal tissue—the first experimental demonstration of the involvement of the anterior pituitary in ovarian function.[24] Their conclusion gained support from other studies carried out in 1927 by Zondek and Aschheim in mice,[32] and by Smith and Engle in rats,[33] showing that the transplantation of even a sufficient number of pieces of infantile pituitary, which have some gonadotropic activity, into juvenile animals would lead to vaginal opening, findings confirmed by the experiments of Lipshütz, Kallas, and Paez.[34] Indirect evidence also demonstrated that when an immature female rat was placed in parabiosis with another immature ovariectomized female,[35] or a gonadectomized adult male or female rat,[36] the non-ovariectomized female underwent precocious puberty, suggesting that whatever factor did trigger sexual maturation did not originate in the gonads, but that the ovaries of immature animals, in spite of their immaturity, were capable of responding to it. At around the same time, Moore and Price,[37,38] while using young, castrated male rats to disprove the idea that ovarian and testicular hormones were mutually antagonistic, proposed that gonadal hormones and secretions of the anterior pituitary operated as a close negative feedback circuit or loop, with gonadal hormones inhibiting the secretion of pituitary factors (related in Dorothy Price's biographical memoir of Carl Moore, 1974[39]). Pfeiffer, using transplants of ovaries and testes in neonatally gonadectomized rats, also came to the conclusion that testicular secretions inhibited the default capacity of gonadectomized rats to display ovarian cycles,[40] results that were interpreted as indicating the sexual differentiation of the anterior pituitary in response to gonadal hormones. Around 1930, McCullagh[41,42] also became interested in testicular physiology, and showed that a urinary extract from male animals, which he called "androtin" (both "testosterone" and "androsterone" already being in use for specific male hormonal extracts by this time), could replace all the functions of the castrated testis, except for pituitary hypertrophy. Like Moore, he concluded that the testis produced two hormones, one corresponding to his "androtin", in reality, testosterone and related substances, and another that he called "inhibin", which inhibited pituitary hypertrophy.

What Moore, Price, and Pfeiffer didn't take into account, unfortunately, influenced as they were by the hypophysiocentric views of Fröhlich, Cushing, and the like, was that the pituitary itself was controlled by another entity—the brain. It was left to Hohlweg and Junkmann[43] to suggest that the hypothalamus was at the apex of what we now know as the "hypothalamic–pituitary–gonadal" (HPG) axis, a suggestion that did not meet with a very warm reception for quite some time. However, Hohlweg himself was merely borrowing an idea put forward in 1909, more than 20 years earlier, by Aschner,[44] and one that had profoundly annoyed Cushing, who believed that the pituitary alone controlled peripheral organs such as the adrenals, thyroid, and gonads. In 1912, in his address to the Royal and Imperial Society of Physicians at Vienna, Aschner concluded by saying "Not only hypophysectomy, but even a mere wound in the base of the thalamencephalon leads to atrophy of the gonads in male and female dogs. The extent to which the hypothalamus can be regarded as a regulatory center for endocrine glands must therefore be decided after further research has been carried out" (quoted by Hohlweg[45]). Cushing, in his "The Pituitary Body and Its Disorders", also in 1912,[46] added as a footnote, "Aschner, it must be confessed, has opposed the view of essentiality of the gland to life. He is inclined to attribute the fatalities to some injury of hypothetical nerve centers of the infundibular region. As Biedl points out, the operative method Aschner employed is open to criticism…". But Aschner's "operative method" in dogs consisted of

passing through the roof of the mouth in order to perform a hypophysectomy without injuring the hypothalamus (reviewed by Anderson and Haymaker, 1974)! In 1927, Smith perfected a similar "parapharyngeal" method to hypophysectomize rats, greatly facilitating future studies aimed at dissociating the effects of the removal of the pituitary alone from the effects of damage to the hypothalamus.[47] Indeed, by 1936, van Dyke, in his Physiology and Pharmacology of the Pituitary Body, was able to list nine animal species and eight methods for hypophysectomizing them, and felt that Smith's method was the best.[48]

The 1930s also saw the first fruits of the contact between Harris and Marshall, already an eminent reproductive physiologist, at Cambridge (for a fuller account of Harris' work, see Ref. 49). Indeed, Marshall, who in his Croonian lecture of 1936 gave an account of Hammond's experiments in domestic animals suggesting that the anterior pituitary controlled sexual maturation,[20] had applied electrical stimuli to the heads of rabbits and found that they activated the reproductive organs by activating the anterior pituitary. Harris carried out similar experiments, in rats and with improved electrical equipment, and showed that electrical stimuli delayed ovulation but induced pseudopregnancy, suggesting that the excitation of "some neural structure" had stimulated the anterior pituitary to act on the ovaries.[50] After the second world war, Harris, using Smith's method for hypophysectomy, went on to show, first with Green[51,52] and then with Jacobsohn,[53,54] that the pituitary portal vessels acted as a "humoral relay" between the central nervous system and the anterior pituitary, that the direction of the portal circulation was brain-to-pituitary (confirming Wislocki's assumptions,[55] and refuting Popa and Fielding's)[56] and that the pituitaries, which were not themselves sexually differentiated, exerted their effects on the gonads only if the hypothalamo-hypophysial vascular connection was intact or had been reestablished. Everett, Sawyer, and Markee[57] and Everett and Sawyer[58] also confirmed that the brain and not the pituitary was the seat of sexual differentiation, and that there was a critical period during which a neurogenic signal could act on the pituitary to induce the release of gonadotropic hormones, and thus trigger ovulation. Based on these and other findings, in his 1955 monograph, "The Neural Control of the Pituitary Gland" (Ref. 59; see also Harris' Henry Dale lecture, published posthumously in 1972[60]), Harris concluded that hypothalamic nerve endings in the median eminence released pituitary-stimulating substances into the portal circulation, and that the time needed to achieve puberty was due not to the immaturity of the pituitary, but to the immaturity of the hypothalamus.

Harris' numerous predictions were confirmed by various groups in the following years, mostly in rats. In 1957, Nikitovitch–Winer and Everett[61] also demonstrated that the gonad-stimulating effects of the pituitary depended on its physical proximity to the hypothalamic median eminence, permitting functional portal circulation. Further, McCann, Taleisnik, and Friedman,[62] Courrier et al.[63] and Nikitovitch–Winer,[64] showed that median eminence peptide extracts could stimulate the release of luteinizing hormone by the pituitary. Conversely, Donovan and van der Werff ten Bosch[65] and Elwers and Critchlow[66] showed, respectively, that rats with a lesioned hypothalamus underwent precocious puberty, indicating the existence of an inhibitory influence of the hypothalamus on the pituitary, and that the hypothalamic stimulation of the pituitary–gonadal axis was itself inhibited by the amygdala—the first evidence of the involvement of an extrahypothalamic region in the process. In the early 1970s, Raisman and Field showed that the hypothalamic preoptic area was indeed sexually dimorphic, with a greater number of dendritic spines in females than in males, an effect dependent on gonadal steroid action in the early postnatal period.[67,68] Although Harris himself died in 1971, the groups of Guillemin and Schally, with the tools and resources to identify Harris' hypothalamic "releasing factors", which had now attained the status of "releasing hormones", used literally millions of sheep and pig hypothalami, respectively, to purify, sequence, and characterize luteinizing hormone releasing factor (LRF) (as it was called then), now usually termed gonadotropin releasing hormone (GnRH). For this monumental work, they shared the Nobel Prize for Physiology and Medicine in 1977, and Schally, in his Nobel lecture, acknowledged the founding contributions of Harris.[69,70]

One aspect of this neuroendocrine axis, however, continued to elude scientists over the years—the influence of gonadal secretions themselves on the hypothalamus and the pituitary. After all, if the chain of command was exclusively "top-down", why was sexual maturation altered in parabiotic rats where one partner was gonadectomized,[35,36] and why did the pituitary become hypertrophied following gonadectomy (an effect noted in 1905 in several species, including the guinea pig, by Fichera[71]) and develop "castration cells" (again, noted as far back as 1914 in humans by Schleidt,[72] in Steinach's laboratory, and in 1917 in rats by Addison[73])? Even more importantly, how was it that minute amounts of ovarian or testicular hormones could prevent the formation of these cells[74,75] or the accompanying effect on puberty in immature animals?[76,77] These knotty questions and the growing realization that the HPG axis was in reality an HPG loop led to the formulation of the "gonadostat" hypothesis by Ramirez and McCann in the mid-1960s[78,79] by analogy to the thermostat. According to this hypothesis, in immature animals, the hypothalamo-pituitary segment of the HPG axis would be sensitive to inhibition by gonadal steroids, but this sensitivity would undergo a

downward shift as the animal approached puberty, with a consequent increase in gonadotropin secretion and the stimulation of further gonadal hormone production, until their levels reached those required for fertility.[80] For the next decade and a half, this hypothesis dominated scientific thought as to the mechanism by which gonadal steroids contributed to the onset of puberty, with much experimental proof provided to support it (reviewed in Ref. 16) and but one dissenting view—that of Morrison and Johnson, suggesting that the restraining influence on gonadotropin secretion originated not from gonadal steroids, but from inhibitory factors present in the hypothalamus in immature animals.[81] It was not until 1981, when Ojeda's group demonstrated that in fact the switch in the sensitivity of the hypothalamo-pituitary unit to negative feedback by estrogen occurred not *before* but *after* the onset of puberty,[82] that the "gonadostat" hypothesis was definitively replaced by our current view of the maturation of the HPG axis.

During these fruitful decades, many of the important strides made in deciphering the mechanisms underlying the hypothalamic–pituitary connection were also due to technical advances: the invention of the radioimmunoassay in the late 1950s by Yalow,[83] which allowed hormone levels in the blood to be accurately measured (and for which she shared the Nobel with Guillemin and Schally), and its adaptation to pituitary gonadotropins by Odell and colleagues[84,85] as well as other groups, and to hypothalamic releasing factors by Naftolin under Harris' mentorship;[86] the invention of the ovarian ascorbic acid depletion method to assay LH levels, used by McCann et al.;[62] a method for collecting portal blood devised by Worthington, and used by Fink in Harris' laboratory to measure GnRH in the portal circulation of rats;[87,88] the invention of Halász' famous knife, which allowed afferents and efferents to the mediobasal hypothalamus to be transected without interrupting the portal circulation,[89] thus permitting a generalized map of hypothalamic regions and their connections to be established;[90] the extraction and isolation of molecules by chromatographic separation using Sephadex columns;[91] and others tools and techniques too numerous to list here. By the end of the 1970s, however, once the presence of GnRH neurons and projections in the hypothalamus, and especially in the preoptic area, had been demonstrated by Barry, with other groups hot on his heels,[92–94] the history of puberty entered a new phase—that of genetic and molecular characterization.

The first step in this direction was an entirely serendipitous one—the discovery by Fink's laboratory in 1977 of a naturally occurring mouse mutant that did not produce functional GnRH: the hypogonadal or *hpg* mouse.[95] By 1986, the genetic abnormality of this very useful animal model in which to study the effects of GnRH was elucidated by the Seeburg group—a deletion mutation in the GnRH gene leading to a lack of GnRH peptide.[96] Although this mouse model, "Nature's Knockout" as it has been called by Gibson et al.,[97] has taught us a lot about the role of GnRH in the establishment of sexual maturation, new tools that allow the expression of genes to be manipulated at the level of specific cells or specific periods of development, the establishment of immortalized GnRH neuronal cell lines, the possibility to trace functional neuronal pathways even beyond the synapse, etc., are leading to ever greater insights into these processes, and model systems in which to study them.[98–101] Among relatively recent developments, it is now known, for example, that GnRH neurons themselves express some nonclassical types of estrogen receptors,[8,102] providing one of the missing pieces of the puzzle posed by the HPG loop. In addition, the identification in 2003 of another master molecule in the regulation of puberty, kisspeptin, by de Roux and colleagues and Seminara and colleagues,[103,104] has led to a veritable spate of papers characterizing its role. And Marshall's recapitulation of the influence of the abundance of food on reproductive activity in humans and animals in his Croonian lecture of 1936 appears particularly prophetic in light of new findings regarding the overlap between hypothalamic cells, molecules, and pathways involved in regulating sexual maturation on the one hand, and those controlling the response to metabolic cues on the other.[105] Moreover, elements discovered in one field, such as the occurrence of adult neurogenesis in the hypothalamus in connection with energy homeostasis[106,107] or the role of those mysterious and plastic glial cells, the tanycytes,[108–110] may well have as yet unsuspected repercussions on the other.[111,112] After all, four decades after Julien Barry first demonstrated the presence of GnRH neurons in the hypothalamus in the very laboratory at Lille from which I am writing, this system still has the power to surprise us!

In this chapter, I will present an overview of the neuroendocrine control of puberty, with a particular focus on rodent models. Briefly, I will discuss the advantages and shortcomings (including species differences) of the use of rats and mice as models, the various components of the GnRH system (the GnRH neurons and their associated cellular network, including both other neurons and various types of glia), its structural and functional maturation in males and females during embryogenesis and in postnatal life leading up to puberty, and mechanisms and factors regulating the expression of the GnRH peptide. It should be noted that in this chapter I will use the terms "gonad-dependent" and "gonad-independent" process to indicate changes that are correlated with gonadal steroid secretion or not correlated with the same, respectively. This terminology will not be restricted to the historical use of these terms to differentiate between the suppression of gonadotropin secretion, is only evident in the presence of steroid (gonad-dependent), from that evident in gonadectomized individuals (gonad-independent). For an excellent discussion of other topics related to puberty in rodents and not dealt

with here for a lack of space, such as a detailed description of the maturation of the gonads, the effect of endocrine disruptors on the onset of puberty, upstream gene networks etc., the reader is referred to the previous version of this chapter by Ojeda and Skinner.[16]

RATS AND MICE AS MODELS

Although the subject of puberty has intrigued clinicians and scientists alike for decades, as we have seen above, the mechanisms that trigger its onset and modulate its progress and completion, and in particular the timely activation of GnRH neurons—the key event that sets in motion the rest of the process—remain an important unsolved mystery. Rodents, especially rats and mice, have been very useful animal models in which to study this developmental process, vital to the survival of mammalian species, and, as indicated by the occurrence of the GnRH peptide and its hypophysiotropic role in all vertebrates (reviewed in Refs 5,113), evolutionarily conserved. Apart from their short life spans, high fecundity, and relative ease of maintenance in a laboratory setting, most of the hormonal, cellular, and molecular mechanisms of reproductive maturation identified in rodents so far are similar to those found in higher mammals. This group includes primates, whose use is restricted by their size, cost, and limited reproductive capacity. Although the rat model has been instrumental in deciphering the physiology of sexual maturation, the mouse model, with the advent of modern genetic tools and techniques, has played a key part in helping us understand the underlying cellular and molecular mechanisms, notably by helping to decode the effects of specific mutations seen in human patients, whose phenotype may not always be due purely to a single genetic change. In addition, in cases where human mutations have not yet been identified, animal phenotypes generated by genetic engineering help in predicting the effects of such mutations in humans, and thus constitute invaluable models for translational research. However, a major caveat to keep in mind when using mice and rats to study puberty and extrapolate the results obtained to humans is that, in contrast to our own species, laboratory rodents have a very short prepubertal period (around 6 weeks versus 10 years in humans) because of their limited lifespan and do not exhibit the so-called "juvenile pause" in gonadotropin secretion and gonadal activity that is characteristic of primates[114,115] (however, see the section on puberty in males). Mice and rats thus have not been considered appropriate animal models in which to investigate the mechanisms underlying this quiescent period of relative hypogonadotropism, which is clearly evident in agonadal primates, including man[116–118] (see also Chapter 32). Although primate species are more amenable to the investigation of this particularly intriguing phenomenon, the daily awakening of the gonadotropic axis seen in rodents at the end of their juvenile development[119] may be a useful model for the study of the transition between the juvenile period and the reactivation of the HPG axis in primates.[115,119]

Species Differences between Mice and Rats

From a developmental perspective, it is important to note that some of the events that occur postnatally in rodents are known to take place during embryogenesis in primates.[120–122] However, even among rodents, the rat and the mouse are two distinct species in spite of their comparable life spans, and their physiology is not strictly identical, at least as concerns the external morphological parameters commonly used to assess the progression of sexual maturation during postnatal development.

Females

In the rat, vaginal opening is used as an external sign of the onset of puberty since it is associated with the first ovulation[123,124] and occurs in most cases at the end of the first proestrus,[123] when animals exhibit a uterus weighing >200 mg and ballooned with fluid, and ovaries with large follicles. Within 24 h of vaginal opening, rats typically present their first vaginal estrus.[123,125] Postmortem, rodents can readily be classified according to the phase of puberty depending upon uterine weight, the presence of intraluminal fluid in the uterus, vaginal opening, and the presence of corpora lutea: criteria originally defined by Ojeda and collaborators.[123] In contrast to vaginal opening in rats, the appearance of the vaginal introitus in mice does not coincide with the first ovulation, i.e., puberty, which can occur more than 20 days later,[126,127] but is rather an indicator of the increase in circulating estradiol levels, which prompts this apoptosis-mediated event.[128] The first estrus in mice, as identified by the daily collection and analysis of vaginal secretions from the day of vaginal opening,[129] is strictly correlated with the ability of the female mouse to become pregnant when housed with a sexually mature male.[130]

Males

The production of motile sperm in males occurs between 40 and 55 days of age both in rats and mice.[131–134] Testicular descent occurs after 15 days of age in both species,[135,136] and sexual maturation can be followed by monitoring the growth of the testis[131,137] and preputial separation.[138] However, although the separation of the prepuce from the glans penis (or balanus) as a result of the cornification of the balanopreputial epithelium occurs between 2 and 8 days before the appearance of motile sperm in the epididymis in rats (i.e., around 45 days of age),[138,139] it happens around weaning in mice (around 21 days of age),[140] and thus only marks an early phase of sexual maturation in the latter species. More recently, the

detection of mouse urinary proteins (MUPs≈20 kDa), whose excretion in the urine is stimulated by testosterone, has also been used as a noninvasive marker of early or delayed sexual maturation in mice.[140,141] Postmortem examination of the reproductive tract, e.g., the weight of the seminal vesicles and prostate, can also provide an indication of circulating androgen levels in animals, which can be correlated to the degree of sexual maturation of the individual under scrutiny.[138,140,141]

PUBERTY AND DEVELOPMENT

The onset of puberty and the regulation of fertility are governed by a complex neural network, primarily in the hypothalamus, that converges onto GnRH-producing neurons, the master regulators of gonadotropin secretion and postnatal gonadal growth and function. The proper development of GnRH neurons, GnRH expression, and GnRH signaling is essential for sexual maturation and the normal functioning of the HPG axis in mammals.[95,96,142,143]

The GnRH Neuron

Embryonic Development

Origin and migration of GnRH neurons. GnRH neurons are peculiar neuroendocrine neurons because, unlike other paraventricular neurosecretory neurons that arise from the neuroepithelium of the third ventricle,[144] they originate from both the neural crest and ectodermal progenitors in the olfactory placode,[145] and subsequently migrate from the nose to the brain during embryogenesis.[146,147] In mice and rats, GnRH cells begin to leave the epithelium of the medial olfactory pit around embryonic day 11.5 (E11.5). They migrate through the frontonasal region in close association with growing fibers of the vomeronasal nerve (in an axonophilic mode of migration[148]), penetrate the rostral forebrain at about E12.5 together with the central process of this nerve, and continue their migration towards the hypothalamic region along a ventral branch of the vomeronasal nerve that projects to the basal forebrain[149–152] (Figure 30.1(A and B)). Finally, the neurons detach from their axonal guides throughout their migratory path, disperse further into

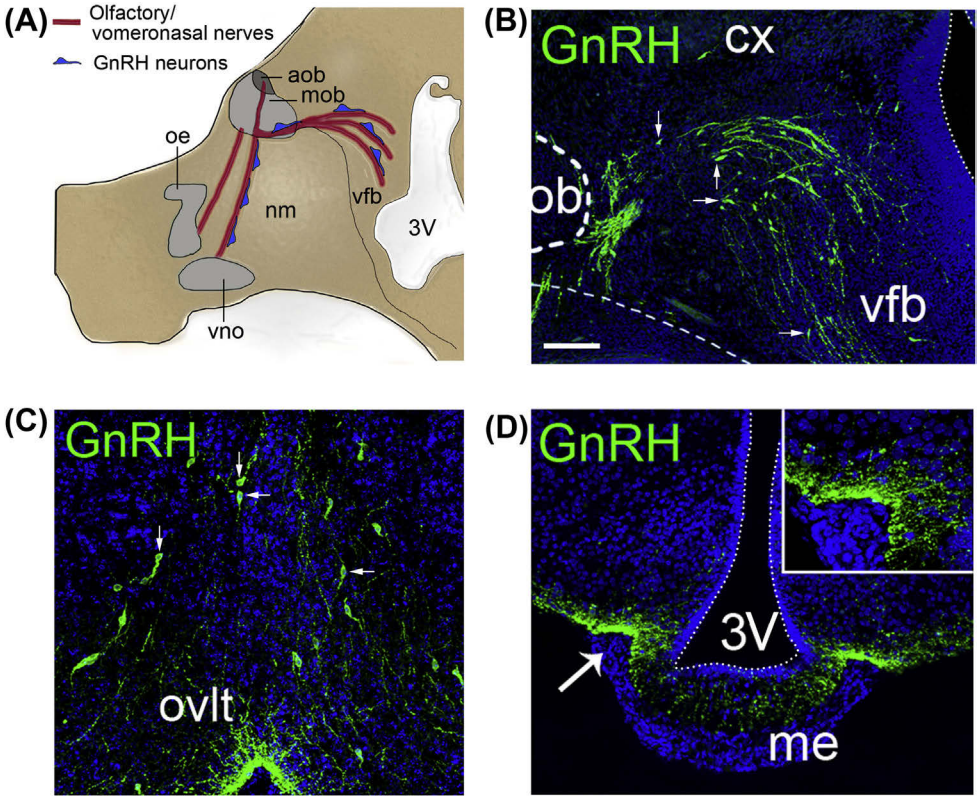

FIGURE 30.1 This figure is reproduced in color in the color plate section. (A) Schematic representation of the head of a mouse embryo at E14.5, showing the scaffold of vomeronasal/terminal nerve fibers (thick lines) along which GnRH cells (neuron-like shapes) migrate from the nose to the brain. (B) Sagittal section of the rostral and ventral forebrain regions at E14.5, immunolabeled for GnRH. Arrows show GnRH neuronal cell bodies. (C, D) Coronal section of the preoptic region showing GnRH neuroendocrine cells (arrows, C) and their nerve terminals in the median eminence (me, D) in newborn (P0) mice. In D, the inset shows a higher magnification of the area identified by the arrow in the main panel. Abbreviations: oe, olfactory epithelium; vno, vomeronasal organ; nm, frontonasal mesenchyme; mob, main olfactory bulb; aob, accessory olfactory bulb; vfb, ventral forebrain; cx, cerebral cortex; ovlt, organum vasculosum of the lamina terminalis; 3V, third ventricle. Scale bar: 100 μm (50 μm in inset).

the brain parenchyma (in particular to the medial preoptic area of the hypothalamus) (Figure 30.1(C)), and stop migrating. At birth, GnRH neurons have reached their final destination, mainly in the hypothalamus, and their projections towards the median eminence and the pituitary portal system are already established (Figure 30.1(D)). Alterations to the birth, migration, or growth and guidance of neurites of GnRH neurons during embryogenesis cause a wide range of malfunctions of the reproductive system, including delayed puberty, subfertility, and even infertility.

Molecular determinants of GnRH neuronal migration. The mouse has been a particularly useful animal model for the study of GnRH neuronal migration and for the identification of some of the genes involved in this process (Table 30.1).

Mutations in many of the genes listed in Table 30.1 are known to be embryonic lethal. However, they provide invaluable information about the natural history of sexual maturation and fertility in mammals. For example, GnRH neurons are completely absent from the noses and brains of mouse embryos with a partial loss of function of the *Fgf8* gene[171] or an invalidation of *Lhx2*,[198] strongly suggesting that these neurons arise exclusively from the nasal region. Indeed, FGF8 is thought to play a role in the fate specification of neurons emerging from the olfactory placode, and its deficiency results in the agenesis of the vomeronasal organs, from which GnRH neurons are first seen to arise during embryogenesis, as well as from the olfactory bulb (see for review Ref. 200). LHX2 is also involved in the formation of the primary olfactory pathways required for vomeronasal nerve development.[198] As expected, the absence of GnRH neuronal migration from the nose to the brain in LHX2-deficient mice, which, in contrast to homozygous FGF8-deficient mice, do not die during development, leads to the nonoccurrence of puberty and to hypogonadotropic hypogonadism.[198] A less severe phenotype (with some GnRH neurons reaching the hypothalamus) is seen in mouse mutants in which vomeronasal projections are only partially disrupted. For example, SOX10-deficient embryos display altered survival of the glia-like olfactory ensheathing cells,[199] which secrete guidance cues key to olfactory/vomeronasal axon development and GnRH neuron chemotaxis.[201] The same is true of mice lacking receptors for NETRIN-1 or SEMA3A (DCC and neuropilin 1, respectively), required for the development of the ventral branch of the vomeronasal nerve, which extends from the olfactory bulb to the presumptive hypothalamic territories and guides GnRH neuronal migration within the brain.[155,164] Interestingly, the few mutant mice with deficient SEMA3A receptor signaling that survive into adulthood (SOX10 and DCC mutants all die around birth[155,199]) exhibit delayed puberty,[164] suggesting that the timing of sexual maturation during postnatal development could be modulated by the number of GnRH neurons that reach the hypothalamus during embryogenesis. However, this idea is brought into question by several studies. For example, Herbison and colleagues have examined in greater detail the relationship between embryonic GnRH neuronal migration and sexual maturation during postnatal development by using the GN23 mouse, in which ephrin signaling is altered by an upregulation of the expression of the receptor EPHA5 in the nasal compartment, migrating GnRH neurons and certain brain regions.[157,202] Excessive EPHA5 signaling appears to inhibit axonophilic GnRH neuronal migration in a dose-dependent manner, as only 12% and 34% of the total GnRH neuronal population usually seen in the hypothalamus of wild-type mice are found in their homozygous and hemizygous GN23 littermates, respectively. Surprisingly, although homozygous GN23 mice

TABLE 30.1 Genes Encoding for a Variety of Proteins Thought to Influence the Initiation, Maintenance, and Cessation of GnRH Neuronal Migration and Thus Pubertal Maturation of the Reproductive Axis in Mice

Adhesion Molecules	Guidance Cues	Growth Factors	G-protein-Coupled Receptors	Neurotransmitter/ Neurotransmitter Synthesis	Transcription Factors
Ncam/PSA-NCAM[153] *β3Gnt1*[154]	Netrin/*Dcc*[155,156] *Epha5*[157] Reelin[158] *Nelf*[a][159,160] ([161,162]) *Sema3a*[a]*/Nrp1/Nrp2*[163–165] ([164,166]) *Sema4d/Plexin-b1*[167] *Sema7a*[a]/β1-intergrin[168,169] ([170])	*Fgf8*[a]*/Fgfr1*[a][171,172] ([172,173]) *Hgf*/cMet[174] *Axl*[a] and *Tyro3*[175] ([176]) *Vegf164*[177]	*Cxcr4/Cxcr7*[178–181] *Prok2*[a]*/Prokr2*[a][182,183] ([183,184])	*Gad67*[185,186] *Cck/Cck-1r*[187]	*Chd7*[a][188] ([189,190]) *Ebf2*[191] Necdin[a][192,193] ([194,195]) *Nhlh2*[196] *Otx2*[197] *Lhx2*[198] *Sox10*[a][199] ([176])

[a]*Genes that have been found to be mutated in patients with hypogonadotropic hypogonadism, with the relevant references indicated in parentheses.* Cck*: cholecystokinin;* Chd7*: chromodomain helicase DNA binding protein 7;* Cxcr*: C-X-C chemokine receptor;* Dcc*: deleted in colon cancer;* Ebf2*: early B cell factor-2;* Epha5*: ephrin A5;* Fgf*: fibroblast growth factor;* Fgfr*: FGF receptor;* Gad67*: 67 kDa isoform of glutamic acid decarboxylase;* β3Gnt1*: β1,3-N-acetylglucosaminyltranferase-1;* Hgf*: hepatic growth factor;* Lhx2*: LIM-homeodomain 2 gene;* Nhlh2*: nescient helix-loop-helix 2;* Ncam*: neuronal cell adhesion molecule;* Nelf*: nasal embryonic LHRH factor;* Nrp*: neuropilin;* Otx2*: orthodenticle homeobox 2;* Prok*: prokineticin;* Prokr2*: prokineticin 2 receptor; PSA-NCAM: polysialic acid-NCAM;* Sema*: semaphorin;* Vegf*: vascular endothelial growth factor.*

are either infertile or markedly subfertile, the onset of puberty is not substantially altered in these mice, despite clear hypogonadism.[157,202] These findings are also strongly supported by the observation that the genetic ablation of more than 90% of GnRH neurons at birth does not delay the onset of puberty.[203] Taken together, these studies suggest that, because of the robustness of the system, any alteration that affects only the number of GnRH neurons reaching the hypothalamus during embryonic development may result in subtle rather than absolute alterations in sexual maturation during postnatal development.

These deductions presuppose a simple genetic background in which the effects of the lack or overexpression of a given gene can be clearly teased apart. In humans, with their heterogeneous genetic background, however, a reduction in the number of hypothalamic GnRH neurons in association with sequence variations in certain genes is thought to be responsible for absent, partial, or delayed puberty.[204,205] The penetrance of these mutations appears to be modulated by an interaction between genetic (presence or not of disease-modifying genes) and environmental factors, and stochastic events (e.g., environmental cues), and modifier genes are often proposed to explain, at least in part, the phenotypic variability observed, as they might influence the expression of a given disorder.[204,205]

Establishment of GnRH neuroendocrine projections. The cell bodies of GnRH neurons are located in a continuum along their migratory pathway, with an especially high concentration in the medial preoptic area of the hypothalamus. In order to stimulate gonadotropin release from the anterior pituitary, the neurohormone needs to be released into capillaries of the pituitary portal system in the median eminence. GnRH neuroendocrine axons are first seen to reach the median eminence by around E15–E16 in rodents.[206–208] An interesting phenomenon in the GnRH system, as in other neural circuits,[209] is that many of the molecular signals controlling the migration of these neurons to their destinations reemerge periodically to play other roles throughout the development of the system. For instance, a gene known to regulate GnRH neuronal migration, such as the semaphorin SEMA7A, may be reused at a later development stage for neurite extension. SEMA7A and its receptor, β1-integrin, regulate GnRH neuronal motility during their journey from the nose to the brain, and their genetic invalidation in mice results in a 30% reduction in the number of GnRH cell bodies in the hypothalamus of adult animals;[168,169] surprisingly, β1-integrin expression in GnRH neurons is also required for neuroendocrine axon extension towards the median eminence.[169] Mice lacking β1-integrin expression selectively in GnRH neurons, in which both GnRH cell migration and axonal extension are disrupted (more than 70% of GnRH-immunoreactive fibers are lost in the median eminence of mutant mice when compared to control littermates), exhibit striking pubertal deficits, such as retarded vaginal opening, a marked delay in first ovulation, and GnRH deficiency in adulthood (disrupted estrous cycles and the inability to generate a normal LH surge).[169] A milder phenotype is observed in mice harboring GnRH neurons with impaired FGFR1 signaling,[210] which has been shown to promote GnRH neurite outgrowth in primary neuronal cultures in both rats[211] and mice.[212] As these processes are studied in greater detail, no doubt more instances of such polyvalent molecules will be uncovered.

GnRH release and signaling. By the time the first neuroendocrine projections arrive at the median eminence, around E15–E16,[206–208] the vasculature of the median eminence already displays the essential characteristics of primary portal capillaries, including an attenuated, fenestrated endothelium, designed to facilitate the free exchange of molecules between the portal circulation and the brain by bypassing the blood–brain barrier (BBB).[206,213] However, it is only by E17–E18 that the first dense-core vesicles carrying the neurohormone reach these neuroendocrine axon terminals[207,208,214] and that the first neurohemal contacts can be visualized.[206] Together, these ultrastructural findings suggest that neurohormone release into pituitary-portal blood vessels occurs during the last 2 or 3 days of fetal life, when hypothalamic GnRH content, pituitary LH content, and GnRH binding sites (the GnRH receptor, GnRHR) are seen to increase dramatically,[215,216] and pituitary cells respond to exogenous GnRH by releasing LH.[217] This early GnRH-mediated activation of pituitary gonadotropes likely plays a key role in the priming of the HPG axis by favoring the formation of functional anterior pituitary endocrine cell networks.[218–220] Indeed, the genetic engineering of mice, in which GnRHR-expressing cells can be ablated under the effect of diphtheria toxin, has demonstrated that the embryonic increase in LH secretion is needed to promote the proper development of FSH-expressing gonadotropes.[221] GnRH stimulation of pituitary gonadotropes activates GnRHR, a G protein-coupled receptor, and induces the secretion of the gonadotropins LH and FSH, in large part through $G_{q\alpha}$ and $G_{11\alpha}$ activation and the subsequent induction of calcium mobilization.[222–224] Although the constitutive activation of GnRHR has been reported to lead to precocious puberty,[225] deleterious mutations in the GnRHR gene have been shown to cause hypogonadotropic hypogonadism with a delay or absence of puberty in both humans[143] and mice,[226–228] without affecting embryonic GnRH neuronal migration.[229]

Postnatal Maturation

Stages of postnatal maturation. As mentioned above, at birth, GnRH neurons have reached their final destination within the hypothalamus, where they are diffusely

distributed and are particularly abundant in the preoptic region in both mice and rats. Their axons target the pericapillary space of the median eminence, where they release their neurohormone into the fenestrated vessels of the pituitary portal blood system for delivery to the anterior pituitary. During postnatal development, GnRH neurons are subjected to a sequence of complex maturational events affecting their biosynthetic capacity, neurosecretory pattern, and morphology, which ultimately lead to sexual maturation and the initiation of puberty. This array of events, which may be linked, at least in part, to the integration of postmigratory GnRH neurons into the neural network responsible for relaying bodily information to these core neurons, has been divided into several phases in both males and females based on morphological and physiological parameters, as delineated earlier by Ojeda and colleagues.[121] In males, postnatal sexual development can be divided into four stages: a neonatal period that comprises the first week of extrauterine life (where the day of birth is designated postnatal day 0 or P0), an infantile period that extends from P8 to P21 (age at weaning), a juvenile period that ends around P35 (in rats), and a peripubertal period that ends at 55–60 days of age with the appearance of mature sperm in the vas deferens. Female postnatal development of the reproductive axis can also be divided into four stages: a neonatal period from birth to P7; an infantile period from P8 to P21; a juvenile period that ends around day 30 in rats and between days 25 and 40 in mice, depending on the strain; and a peripubertal period that is variable in duration but culminates with the occurrence of the first ovulation.

Hypothalamic expression of the GnRH gene. The expression of the GnRH gene appears to be required neither for GnRH neuronal migration nor for GnRH neuronal maturation, since the distribution of GnRH neurons in *hpg* mice harboring a naturally occurring deletion of about 33.5 kb encompassing the distal half of the gene encoding the GnRH precursor is similar to that in wild-type mice,[96,229] and the lack of the GnRH peptide does not preclude the spontaneous generation of bursts of action potentials in GnRH neurons.[229] However, decreased brain GnRH expression caused by the deletion of an enhancer region in the GnRH promoter that is critical for its expression in the hypothalamus,[230] or by the selective deletion of some kinases, such as JAK2 (Janus-activated kinase 2) in GnRH neurons,[231] has been shown to be associated with a significant delay in the onset of puberty and abnormal estrous cyclicity in adults.[231,232] Even though the overall impairment of fertility in these mice varies widely, from mild[232] to severe,[231] these two studies suggest that the proper regulation of enhancer/repressor elements in the GnRH promoter is required for the timely completion of female sexual maturation. Insights into the molecular mechanisms that regulate the expression of the GnRH gene by acting directly on its promoter have mostly been gained by studies performed in the immortalized murine GnRH-secreting cell lines GT1-7[99] and GN11[233] in vitro (Table 30.2). In contrast, few, if any, studies have been undertaken to determine whether these regulators play an actual role in the control of the GnRH promoter in vivo, and whether they are important for the normal physiological events associated with reproduction, including puberty, estrous cyclicity, and fertility. One study has shown that mice in which GnRH neurons are selectively lacking in the expression of CREB (cAMP response element-binding protein), a repressor of the GnRH promoter in vitro,[252,253] undergo normal puberty,[254] suggesting that this transcription factor does not play a major role in the postnatal maturation or function of GnRH neurons in vivo. In contrast, another study has shown that the selective invalidation in vivo of the expression of OTX2 (orthodenticle homeobox 2), a well-known activator of GnRH gene expression,[243–245] in GnRH neurons, results in a marked decrease in GnRH mRNA expression in the hypothalamus of adult mice as well as the delayed onset of puberty. Unexpectedly, however, this decrease appears to be due to the premature death of GnRH neurons during embryonic migration in these mice.[197] However, the use of these mice has enabled the demonstration that OTX2 expression is required to mediate the in vivo increase in GnRH gene

TABLE 30.2 Factors Thought to Influence the Transcriptional Activity of the GnRH Gene in Rodents

Activation	Repression	Inhibition of repression
POU2F1[234–238]	MSX1[239,250]	AES[245,250]
DLX1/2/5[239,240]	CEBPB[234,251]	
PBX1[241,242]	TLE4[245,250]	
PKNOX1[242]	ERβ1[249]	
MEIS1[241]	FOS[252,253]	
OTX2[197,243–246]	CREB[252,253]	
EGR1[247,248]		
ERβ1[249]		

AES, amino-terminal enhancer of split; CEBPB, CCAAT/enhancer-binding protein beta; CREB, cAMP response element-binding protein; DLX, drosophila distal-less-related gene; EGR1, erythroblast transformation-specific (ETS)-related gene; ER β1, estrogen receptor beta 1; FOS, FBJ murine osteosarcoma viral oncogene homolog; MEIS1, myeloid ecotropic viral integration site 1 homolog; MSX1, msh homeobox homolog 1; OTX2, orthodenticle homeobox 2; PBX1, pre-B-cell leukemia homeobox 1; PKNOX1, PBX/knotted 1 homeobox 1; POU2F1, POU class 2 homeobox 1; TLE4, transducin-like enhancer of split 4.

expression induced by kisspeptin,[246] another primarily hypothalamic peptide that potently stimulates GnRH neurons and whose action is essential for puberty and subsequent fertility.[255]

Postnatal changes in hypothalamic GnRH content. In both male and female rats and mice, hypothalamic GnRH content increases steadily during the neonatal period.[215,256–259] Although it remains constant in the first part of the infantile period, between 8 and 12 days of age, it subsequently increases sharply between P12 and P16, and continues to increase during the juvenile period to reach a maximum during the peripubertal period in females.[95,258,259] In males, however, GnRH content does not peak but continues to increase throughout adulthood.[95,256] Interestingly, in vitro experiments using retrochiasmatic hypothalamic explants containing GnRH axons and nerve terminals, but probably few cell bodies [260] from male and female rats, have shown that GnRH is secreted in a pulsatile fashion from postnatal day 5 to puberty.[257,261] Also, changes in pulsatility have been shown to differentially regulate gonadotropin secretion both in vitro[262] and in vivo.[263]

FEMALES: the pattern of postnatal GnRH secretion, changes in circulating gonadotropins, and the recruitment and maturation of the first ovarian follicles (Figure 30.2).

Neonatal period. As mentioned previously, and as indicated by its name, the ultimate function of GnRH is to stimulate the secretion of the gonadotropins FSH and LH, and thus influence gonadal development and function. In females, the ovary at birth consists of cords and oogonia.[122] Primordial follicles are formed by 3 days after birth, whereas well-developed secondary follicles are seen by P7.[264–267] The activity of the hypothalamic–pituitary unit does not appear to be required for ovarian development during this first week of postnatal life, since follicular development is seen to be normal in GnRH-deficient *hpg* mice at 7 days of age.[268] In addition, FSH and LH are unlikely to exert any direct effect on the primordial follicles because they still lack a functional gonadotropin receptor signaling mechanism.[269–272] Instead, primordial follicles may respond to activators of the cAMP pathway that are released by sympathetic nerves, which develop before the initiation of folliculogenesis[273–275] or other paracrine factors produced within the ovary (see Chapter 21)

Infantile period. During the second and third weeks of life, when the first wave of ovarian follicles, which will ovulate at puberty, have reached the secondary stage and become responsive to FSH,[269–272] key neuroendocrine events prompt them to develop into preantral and then antral follicles over the next 3 weeks.[264–267] At the beginning of the infantile period, circulating FSH levels rise dramatically and reach peak values at 12 days of age in both rats[276–279] and mice.[259,280] This infantile surge in FSH enhances the development of preantral follicles and rescues early antral follicles from apoptotic death.[122] Blunting this infantile rise in FSH levels by GnRH antagonist treatment decreases ovarian weight and preantral follicle development in rats at P19, whereas FSH treatment rescues these effects.[266] In addition, FSH-deficient mice are infertile due to the lack of maturation of secondary or preantral follicles into antral follicles.[281] Along the same lines, the absence of circulating FSH in *hpg* mice (which lack GnRH), is associated with a dramatic reduction in the number of follicles reaching the preantral stage and the complete absence of ovarian follicles attaining the antral stage.[268] Similarly, the alteration of the course of the infantile FSH surge in transgenic mice, which exhibit hypothalamic defects in GnRH release, results in delayed puberty and reduced fertility in primiparous females.[259] Even if their fluctuation is less consistent during this period, LH levels are also elevated in both infantile rats and mice,[259,277,282] and sporadic bursts of secretion have been reported to occur.[277,283] However, LH does not appear to play a major role in ovarian development at this early stage of sexual maturation, since LH receptor knockout mice, in contrast to FSH knockout animals,[281] exhibit early antral follicles.[284]

How do these developmental changes in circulating gonadotropin levels relate to GnRH release during the infantile period? Both early and more recent in vitro experiments, which measure GnRH release from hypothalamic explants by using radioimmunoassay, have suggested that the frequency of GnRH pulses increases throughout sexual maturation.[261] Indeed, GnRH pulses occur every 90 min at P5, every 60 min at P15, and every 30 min during the juvenile and peripubertal periods in rats.[257,261] Thus, this provides a tentative explanation as to why the secretion of FSH during the infantile period is so different from that of LH: unlike high-frequency GnRH pulses, which promote LH release, low frequency GnRH secretion in infantile animals has been shown to induce high levels of FSH secretion,[262,263] which are at least five times greater than in peripubertal animals. These high levels of FSH may also be linked to the responsiveness of the pituitary to GnRH, which appears to be much higher in infantile rats than in animals at later developmental stages,[285–287] as well as to the lack of negative feedback onto the brain.[288,289] It is worth noting in this context that between 7 and 21 days of age, there is an overall increase in steroidogenic enzyme activity in the ovary. This leads to, among other results, a marked and continuous increase in the production of estrogens, thought to result from an interaction between FSH and LH signaling within the infantile ovary in a manner similar to that seen in adult animals.[290,291] Indeed, this developmental change requires gonadotropin stimulation, since ovarian metabolism in adult GnRH-deficient *hpg* mice is similar to that in normal mice at 7 days.[292] However, because of the presence of the estradiol-binding α-fetoprotein (AFP) in the circulation,[293,294] whose levels remain high until

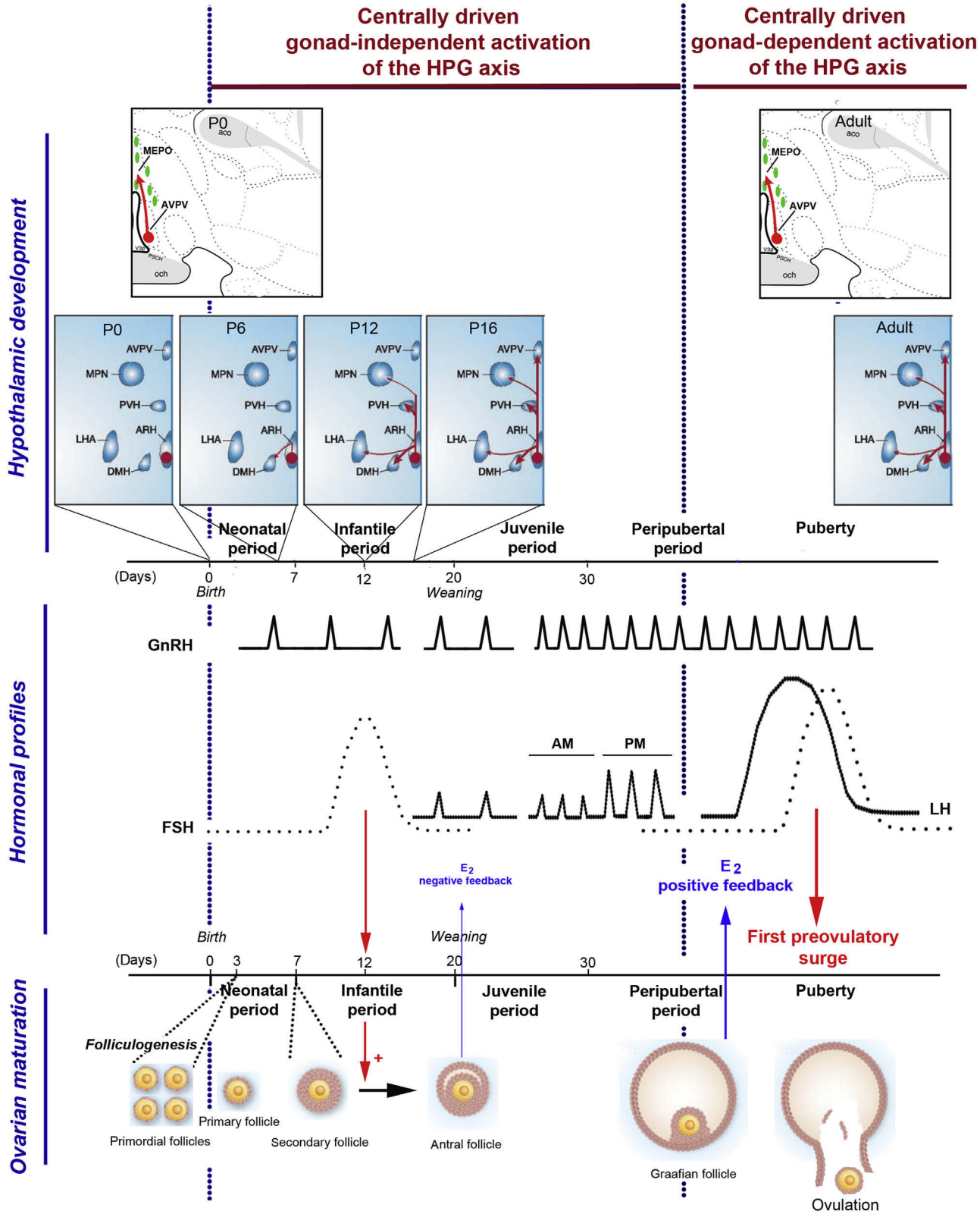

FIGURE 30.2 Centrally driven gonad-independent and gonad-dependent activation of the female HPG axis. (*Hypothalamic development*) Although the morphological development of the hypothalamic site (the AVPV) known to mediate the positive-feedback effect of estradiol (E_2) onto GnRH neurons (ovals) in sexually mature female individuals is complete at birth, axons of the ARH neurons, which are suspected of playing a key role in mediating the estrogen negative feedback, first reach the preoptic region during the infantile period. Mature projections are established by postnatal day 16 (P16). (*Hormonal profiles*) Schematic diagrams illustrating our present understanding of centrally driven gonad-independent and gonad-dependent changes in hormonal profiles during female postnatal development. (*Ovarian maturation*) Schematic diagram illustrating how the aforementioned changes affect folliculogenesis, and conversely, how the advancement of follicular maturation modulates hypothalamic/pituitary function through E_2 secretion during postnatal development. AVPV, anteroventral periventricular nucleus; ARH, arcuate nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; MEPO, median preoptic nucleus; MPN, medial preoptic nucleus; PVH, paraventricular nucleus.

P16[293–295] to protect the female brain from masculinization and defeminization,[296] the negative feedback of ovarian estradiol onto the hypothalamus, which could reduce GnRH secretion and thus limit circulating FSH, is relatively ineffective during the infantile period,[288,289] even if it becomes operational as early as P12-P16 in mice.[297] In summary, the infantile period constitutes a key stage of postnatal development during which the first centrally driven, gonad-independent activation of the HPG axis occurs.

Juvenile period. By the end of the infantile period, at weaning, together with the steady decrease in peripheral AFP concentrations[298] and constant ovarian growth,[282] circulating FSH levels decrease to nadir values, the sporadic bursts of LH release described above disappear, and plasma LH levels remain low.[277,282,283] GnRH pulse frequency in hypothalamic explants is, however, seen to increase significantly to reach adult values, i.e., about one pulse every 30 min,[261] a frequency known to maximally stimulate LH secretion both in vitro[262] and in vivo.[263] In accordance with these data, the pattern of LH secretion, which is distinctly pulsatile and accelerates during the latter part of the juvenile period,[119,299–301] is also seen to exhibit interpulse intervals of about 30 min in vivo.[119] These findings suggest that despite the constant increase in steroid negative feedback on gonadotropin release, which becomes maximally effective during the juvenile period,[298,302–304] GnRH neuroendocrine output matures.

In line with this notion, although proestrous levels of exogenous estradiol fail to promote LH release during the infantile period, they become capable of doing so when animals enter the juvenile period, and sensitivity to preovulatory levels of estradiol increase dramatically with age to reach a maximum at the end of the juvenile period, i.e., around P30.[288] This stimulatory effect of estradiol on LH release no doubt involves the activation of hypothalamic GnRH secretion, as gonadal-steroid-induced LH surges in juvenile rats have been shown to occur concomitantly with an increase in GnRH release into pituitary-portal blood vessels.[305] In summary, during the juvenile period, the hypothalamic–pituitary axis becomes sensitive to the low levels of estradiol produced by the ovaries, thus enabling the development of negative feedback by estradiol. In parallel, the hypothalamic–pituitary axis acquires the ability to respond to high exogenous estrogen levels, although the ovarian follicles at this stage of development do not produce sufficient amounts of estradiol,[306] or for sufficient periods of time, to exert a positive feedback effect and stimulate a surge of LH.

Peripubertal period. The transition between the juvenile and the peripubertal periods is marked by the appearance of morning–afternoon differences in the serum concentration of LH.[119,307] This diurnal pattern of release is established in female rats around P30 and is characterized by an increase in both basal LH levels and LH pulse amplitude in the afternoon, whereas LH pulse frequency remains unchanged between the morning and the afternoon.[119,299] Importantly, this phenomenon does not appear to be driven by the gonads, since higher plasma LH levels in the afternoon are also seen in peripubertal rats ovariectomized shortly before measurements.[308] In parallel to this centrally driven, gonad-independent activation of pituitary gonadotropin secretion, some peripubertal animals also exhibit a midafternoon episode of more sustained LH secretion, defined as an LH minisurge.[119] In contrast to the afternoon increase in LH pulse amplitude, these minisurges of LH secretion appear to be caused by subtle increases in serum estradiol levels.[309] In vitro experiments in which peripubertal ovaries are perfused with an LH regimen designed to mimic either morning–afternoon pulses of LH secretion, or LH minisurges, demonstrate that these peripubertal changes in the pattern of LH release are physiologically relevant for the functional development of the ovaries, and presumably stimulate them to produce more estradiol and progesterone.[310] In turn, minisurges in LH secretion evoked by subtle changes in estradiol levels[309] would then lead to further ovarian activation.[310]

Puberty: the first preovulatory surge of gonadotropins and the first ovulation. The ovary's acquisition of the capacity to secrete high levels of estrogens over a period of about 24 h[311] represents the key event in the determination of the timing of the first preovulatory GnRH/LH/FSH surge, i.e., puberty.[123,305] Early immunoneutralization experiments have demonstrated that rising blood estrogen levels (resulting from the stimulation of the ovaries of sexually immature rats with pregnant mare serum gonadotropin [PMSG]) constitute the trigger that activates the release of proestrous levels of LH.[312] In the hypothalamus, this trigger evokes the discharge of GnRH neurons, releasing the neurohormone;[305] in the pituitary, it sensitizes gonadotropes to the stimulatory effect of GnRH.[313] Besides estrogens, progesterone, whose levels also increase dramatically on the day of the first proestrus,[314,315] appears to have a role in facilitating the stimulatory effects of estrogens on GnRH release.[316] In mice, treatment with estradiol, progesterone, or both, is also known to promote pubertal ovulation in sexually immature individuals.[317] In contrast to the infantile, juvenile, and peripubertal periods of sexual maturation, where changes in gonadotropin secretion appear to be gonad-independent, the onset of the first preovulatory surge of gonadotropins, the landmark of puberty, thus appears to result from a centrally driven gonad-dependent activation of the HPG axis (Figure 30.2), as occurs on proestrus in adults.

MALES: the relationship between the pattern of postnatal GnRH secretion, changes in circulating gonadotropins, and testicular maturation (Figure 30.3).

Neonatal period. As seen in females for ovarian growth, the activity of the hypothalamic pituitary axis does not appear to be required for testicular development during the neonatal period. Intriguingly, testosterone during the fetal and neonatal periods, essential for male sexual differentiation, is produced by fetal Leydig cells, which, in contrast to the Leydig cells that develop postnatally, do not require LH for their proliferation or differentiation. Instead, they are completely dependent on placental chorionic gonadotropin.[318–321] The sexual differentiation of male *hpg* mice is normal, despite the nearly complete lack of gonadotropins.[322] The testes of newborn mice deficient in GnRH or FSH, or harboring mutations in the LH receptor, are similar in size, appearance, and function to those of their wild-type littermates, and their intra-abdominal location adjacent to the urinary bladder is the same as in wild-type males.[95,281,284,323] However, postnatally, germ cells in GnRH- or LH-receptor-deficient mice fail to progress beyond early meiosis (the diplotene stage)[95] or are arrested at the round spermatid stage,[284] and the testes, which remain very small, never descend into the scrotum in these infertile mice.[95,284] In contrast, in FSH-deficient mice, spermatogenesis appears grossly normal and males eventually reach puberty and are fertile.[281] During the neonatal period in rats, circulating FSH levels appear to be high up to postnatal day 5, when they drop dramatically[283,324,325] (Figure 30.3), whereas circulating LH levels remain constantly low or undetectable.[283,324,325]

Infantile, juvenile, and peripubertal periods. Although data about putative changes in LH levels during the infantile period in rats are scarce and inconsistent (see for review Ref. 16), it is clear that circulating FSH levels rise dramatically after the second week of life and reach a maximum between 30 and 40 days of age, then decrease when serum testosterone concentrations attain levels similar to those seen in adults both in rats[277,282,283,324–327] and mice.[140,328] The key role of infantile/juvenile GnRH release in male sexual development has been shown using passive immunization[329–331] and treatment with a GnRH antagonist,[332,333] which result in delayed puberty, the permanent impairment of testicular function and defective adult sexual behavior. Concerning the pattern of GnRH release, in vitro studies in the rat have shown that GnRH pulse frequency gradually increases during the juvenile period (0.1 pulse/h) to reach peripubertal values (0.3 GnRH pulses/h).[257] Whether similar changes occur in the mouse is not known. However, the recent development of fast-scan voltammetry to measure GnRH release in real time from brain slices[334,335] suggests that the picture is more complex than believed previously. For example, in mouse brain slices, the pulse frequency of GnRH release has been reported to be much higher in 7–10-day-old infantile than in 40-day-old peripubertal male mice,[335] and this phenomenon appeared to be paralleled by changes in the frequency of GnRH neuron firing.[335] While as suggested by Moenter and colleagues,[335] the pulse frequency of GnRH release could in fact be high during the early infantile period (3.1 GnRH pulses/h), decrease abruptly during the late infantile period (0.1 pulse/h),[257] and gradually go back up to peripubertal values (0.3 GnRH pulses/h)[257,335] during the juvenile period. This possibility is still in the realm of speculation for the moment in the absence of advanced technology capable of permitting longitudinal studies in vitro. Nor is it known whether such a putative triphasic pattern of frequency regulation is due solely to gonadal steroid negative feedback, or whether it is homologous to the hiatus in GnRH secretion seen in nonrodents/higher mammals during the juvenile period. This is an exciting possibility that requires further investigation.

Genetic mouse models in which the signaling pathways of GnRH, LH, or FSH are mutated or harbor gain-of-function mutations, have also added to our understanding of the role of LH and FSH in the control of testicular growth and spermatogenesis.[140,226–228,281,336–342] From these studies, it is clear that changes in the secretion of gonadotropins precede the maturation of the testes. FSH binds to Sertoli cells within the seminiferous tubules to promote their proliferation, which influences spermatogenesis on a quantitative level, but is not essential for fertility.[281,336,337,339] LH stimulates testosterone secretion by acting directly on the Leydig cells that develop postnatally, around day 7 in mice, and whose proliferation and differentiation are also highly dependent on LH.[319] Testosterone is the most important androgen produced by the gonads in males, as it plays pivotal roles in spermatogenesis, the differentiation and maintenance of accessory sex organs and sexual behavior,[343–345] and potentiates the pituitary response to GnRH.[346] Interestingly, testosterone exerts a negative feedback effect on the hypothalamic–pituitary axis throughout life, even during the neonatal period, as shown by experiments in which orchidectomy leads to an immediate increase in gonadotropin secretion that can be suppressed equally readily by the administration of exogenous testosterone.[79,347–350]

Puberty: presence of motile sperm in the epididymis and capacity for sexual reproduction. Spermatozoa in the cauda epididymis–vas deferens junction are first observed around 40 and 55 days of age in mice[131,132] and rats,[133,134] respectively. In line with the fact that the first spermatozoa to appear are few in number and exhibit poor motility and aberrant morphology, and that these characteristics are normalized with age,[132,351] the incidence of fertile matings in mice, for example, increases steadily from 40 to 55 days of age.[131] All these events occur concomitantly with a steep increase in testosterone levels,[140,325,328] whose pattern of secretion has been shown

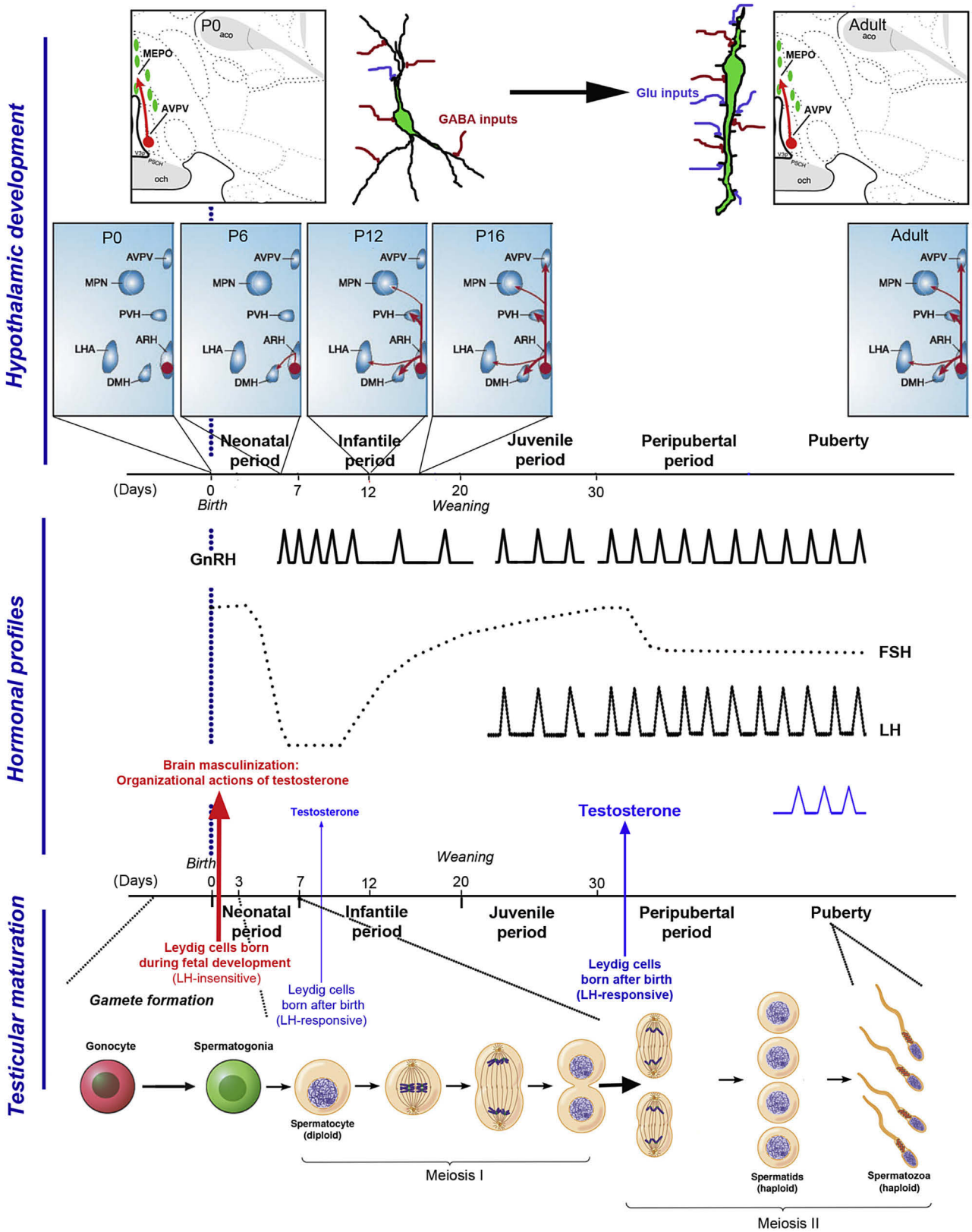

FIGURE 30.3 Postnatal maturation of the male HPG axis. (*Hypothalamic development*) As in females, although development of the sexually dimorphic AVPV is complete at birth and its masculinization via gonad-dependent mechanisms occurs during the neonatal period, ARH projections to the preoptic region develop and mature during the infantile period. In the male, GnRH neurons (ovals) are subjected to extensive pruning of their dendrites and changes in dendritic spine density during postnatal development; although GABAergic inputs to GnRH neurons do not appear to change, glutamatergic afferents are thought to increase dramatically between the infantile period and adulthood. (*Hormonal profiles*) Schematic diagrams illustrating our present understanding of centrally driven (and likely gonad-independent changes in hormonal profiles during male postnatal development). (*Testicular maturation*) Schematic diagram illustrating how the aforementioned changes affect spermatogenesis and Leydig cell function in the testes. Glu, glutamate; AVPV, anteroventral periventricular nucleus; ARH, arcuate nucleus of the hypothalamus; DMH, dorsomedial nucleus of the hypothalamus; LHA, lateral hypothalamic area; MEPO, median preoptic nucleus; MPN, medial preoptic nucleus; PVH, paraventricular nucleus.

to be highly correlated with pulses of LH secretion[352] (Figure 30.3). It is worth noting that in mice and rats, 40–54 days are required to complete the initiation of spermatogenesis, a time period that corresponds exactly to the age at puberty in these species.[353] Spermatogenic development in advance of the normal age of onset of puberty cannot be achieved with precocious gonadotropin stimulation. For example, although mice harboring a common activating mutation of the human LH receptor (D582G), which in boys causes precocious puberty,[318,354] exhibit signs of accelerated sexual maturation such as early preputial separation, spermatogenesis is not advanced, i.e., there is no true precocious puberty.[140] However, premature expression of the androgen receptor in the Sertoli cell of mice accelerates maturation of this critical somatic cell and advances spermatogenesis[328] (see Chapter 16). In humans, approximately 64 days are required for undifferentiated spermatogonia to differentiate to spermatozoa,[355] and this process is not initiated until the onset of puberty is reached after the age of 10 years.

Structural Maturation of the GnRH Neuron

Over the past two decades, several studies have suggested that the structural remodeling of GnRH neurons accompanies developmental changes in their function. Early immunocytochemical studies have shown that the number of GnRH neurons bearing spine-like processes[356,357] increases during postnatal development in both male and female rats.[358,359] Recent studies by Herbison and collaborators, using biocytin filling of green fluorescent protein (GFP)-tagged GnRH neurons in acute brain slice preparations,[360] have extended these findings by showing that the spine density in GnRH neurons increases twofold between the second week of postnatal life and adulthood (when GnRH pulse frequency is thought to increase dramatically,[257,261] see Figure 30.3), both at the level of the somata and of the proximal dendrites in male transgenic mice.[361] Whether similar phenomena occur in females during postnatal development is not known. However, in adult female mice, estrogens have been shown to play an important role in regulating dendritic spine plasticity in GnRH neurons during the ovarian cycle,[362] an effect that may require estrogen receptor β (ERβ) and CREB signaling pathways in GnRH neurons themselves.[254,363] Herbison and colleagues have also shown that in addition to changes in spine density, a specific subset of GnRH neurons is subject to a biphasic pattern of remodeling of the dendritic tree during postnatal development.[361] Indeed, unlike GnRH neurons in the medial septum, GnRH neurons in the preoptic area, which in females are specifically involved in the generation of the GnRH surge at proestrus,[364–366] exhibit an expansive and highly branched dendritic tree during the neonatal period (between P0 and P10). This tree subsequently undergoes dramatic pruning before the peripubertal period (P35) to yield the relatively simple phenotype previously described for mature unipolar or bipolar GnRH neurons.[367] In sexually mature animals, bipolar GnRH neurons extend dendritic processes up to 1 mm in length,[360,368] and sometimes even reach brain sites outside the BBB, such as the organum vasculosum of the lamina terminalis (OVLT)[369–371] and the median eminence.[372–374] In the latter, a recent study has suggested, provocatively, that GnRH neuronal processes could even share both axonal and dendritic functions.[372] For further discussion of the GnRH neuron, the reader is referred to Chapter 11.

The GnRH Neural Network

As the final common target for the central control of reproduction, the activity of GnRH neurons is regulated by a complex array of excitatory and inhibitory transsynaptic and nonsynaptic inputs[14,375–379] (Figure 30.4). This dynamic neural network, which converges onto the GnRH neurons and is likely subject to the direct and indirect influence of a plethora of internal and external signals, matures throughout the developmental period leading to puberty.[16,380,381] Thus, some of these communication pathways are established as soon as the GnRH neurons reach the hypothalamus, whereas others may only appear during postnatal development.[382,383]

The complexity of the neuronal network synaptically connected to GnRH neurons, originally suspected from studies that used classic neuroanatomical techniques to trace neuronal inputs to the medial preoptic nucleus in rats,[384–389] has recently been confirmed using advanced genetic strategies in mice.[98,100,366] Indeed, these latter studies have revealed that neurons from most areas of the hypothalamus, including the anteroventral periventricular nucleus (AVPV) and other preoptic nuclei; the arcuate, ventromedial, and dorsomedial nuclei of the hypothalamus (ARH, VMH, DMH); the lateral hypothalamus (LA); the ventral premammillary nucleus (PMv); and the suprachiasmatic nucleus (SCN); as well as those from the main and accessory olfactory bulbs and the brain stem, synapse directly with GnRH neurons in both males and females. The sequence of establishment of these connections and their maturation are likely to play a major role in the timely unfolding of the developmental program leading to puberty.

Embryonic Development

Most neuronal populations seen to synapse with GnRH neurons in adults[98,100,366] are born during embryogenesis.[144,390–392] For example, hypothalamic neurons,

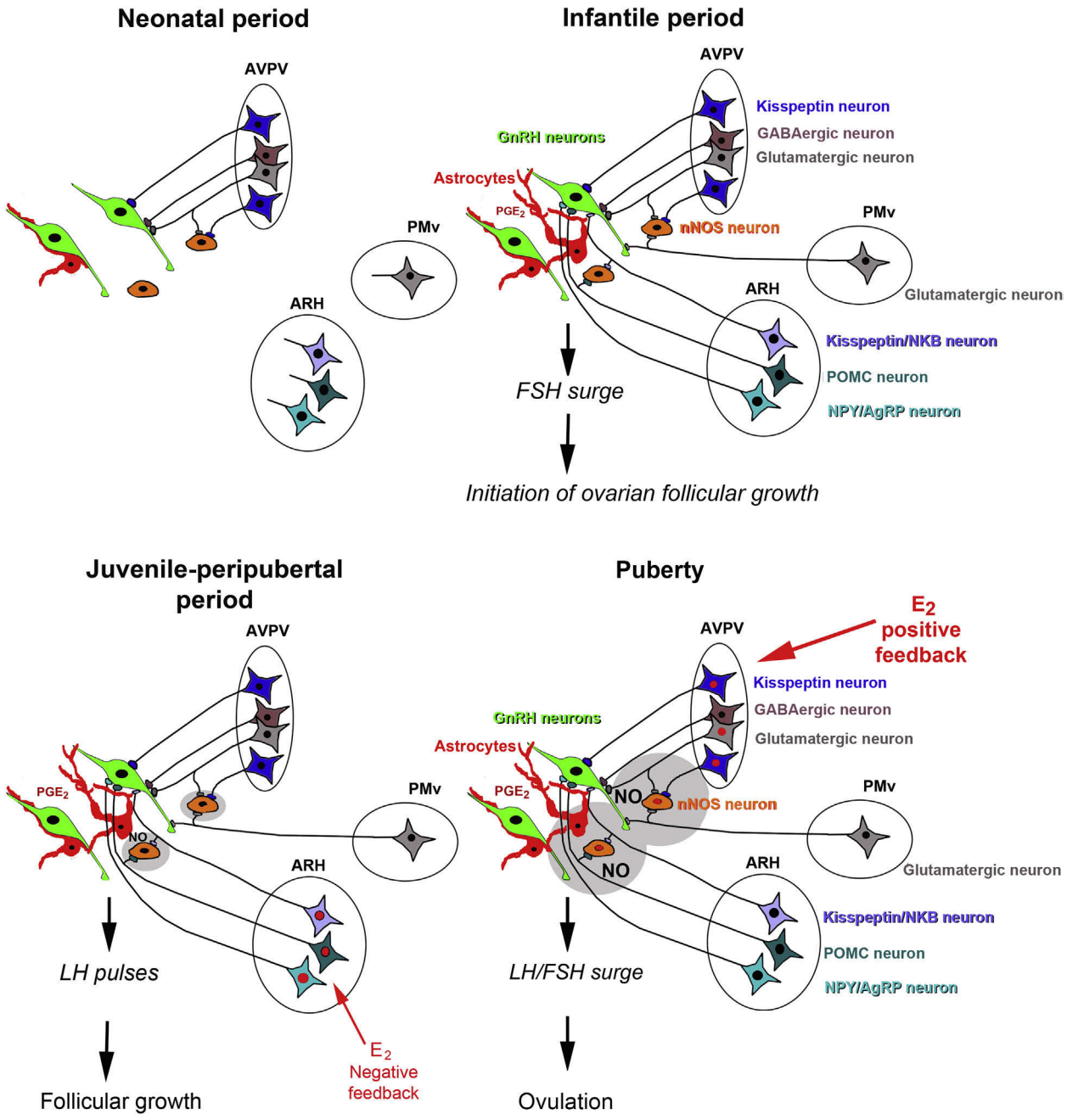

FIGURE 30.4 Schematic diagram illustrating the different maturational steps leading to the onset of puberty in females and their putative neural control during postnatal development. The projection of ARH neurons to the preoptic region during the infantile period and the maturation of the synaptic contacts they establish may constitute the first centrally driven gonad-independent trigger for sexual maturation, by prompting the infantile FSH surge and thereby initiating the growth of the follicles that will ovulate at puberty. Subsequently, with the gradual decrease in circulating levels of alpha-fetoprotein, the low estradiol (E_2) levels produced by growing follicles progressively gain access to the hypothalamus, where they exert a negative-feedback effect on ARH neurons. Besides, neurons and glial cells from the tuberal and preoptic regions of the hypothalamus, and possibly also neurons from other brain areas, contribute to the maturation of the pattern of pulsatile LH secretion throughout the juvenile and peripubertal periods, further promoting follicular growth. When ovarian follicles reach the Graafian stage, the increasing amount of E2 they produce exerts a positive-feedback effect on the neurons of the AVPV, which coordinate the onset of the first preovulatory GnRH and LH/FSH surge, thus triggering the first ovulation and conferring fertility on the individuals. PGE_2, prostaglandin E_2; AVPV, anteroventral periventricular nucleus; ARH, arcuate nucleus of the hypothalamus; PMv, ventral premammillary nucleus.

which are generated predominantly from the epithelium of the third ventricle, are born between E12 and E14 in mice[393–395] and between E12 and E17 in rats.[396–399] Subsequently, these neurons migrate from their site of origin to their final position, a process that is also mostly complete before birth.[400] Two recent thought-provoking studies have demonstrated, using advanced genetic tools to perform toxin-mediated "cell knockouts" in mice, that during embryogenesis and the early neonatal period, the hypothalamus is endowed with a high degree of plasticity, and is capable of coping with the loss of a key neuronal population by developing network-based compensatory mechanisms. Indeed, the ablation of kisspeptin neurons or orexigenic neurons expressing neuropeptide Y/agouti-related protein (NPY/AgRP), two neuronal cell types key to the central control of fertility and food intake, respectively, has no major effect on the physiology of these mice.[203,401] However, the ablation of these neuronal populations later on in life, such as during adulthood, causes infertility and death of the animals due to starvation, respectively.[203,401] Concerning the kisspeptin system, the phenotype seen in the "cell knockout" mice is in striking contrast to that observed in "gene knockout" (or mutant) mice[255] and humans,[402] both of which display an absence of puberty and infertility. One could argue that in the former mouse model, the ablation of kisspeptin neurons is not complete, and that suboptimal numbers of surviving neurons could nevertheless be sufficient to drive the entire system,[403] as is the case with GnRH neurons themselves.[203] However, if this were the case, one could ask why neuronal deletion is complete in adults,[203] leading to infertility, but incomplete in neonates, with the rescue of some residual fertility. An alternative explanation for this remarkable difference is that in the "cell knockout" situation, kisspeptin neurons are appropriately integrated into the GnRH neuronal circuit, as shown by the correct expression of the *LacZ* reporter gene in mutant mice,[255] and likely continue to release the other neuropeptides and neurotransmitters that they express, including neurokinin B, dynorphin,[404–408] and dopamine.[409] In the absence of kisspeptin secretion from these otherwise active neurons, the function of the GnRH neuronal network is thus dramatically disrupted, whereas the total loss of kisspeptin neurons in the "cell knockout" model, and the concomitant absence of all signaling molecules that they made, initiates compensatory adaptations in the neonate.

Postnatal Development

Establishment of afferent projections to GnRH neurons. In both rats and mice, the hypothalamus is still relatively immature at birth, despite the fact that the GnRH neurons themselves and their projections towards the portal circulation in the median eminence are already in place, and continue to develop during the first 2–3 weeks of postnatal life. Indeed, while neurons of the AVPV, the hypothalamic site known to mediate the positive feedback effect of estradiol in mature females,[366,410,411] have completed their projections to the median preoptic nucleus (MEPO) and contact GnRH neurons by birth.[412] The axons of ARH neurons, which are suspected of playing a role in mediating estrogen and testosterone negative feedback[410,413–415] and in the control of pulsatile GnRH secretion,[405,414] first reach the MEPO at P12 and only achieve their final distribution by the end of the infantile period in both males and females,[416,417] when circulating FSH levels dramatically rise[140,276,280,282] (Figures 30.2 and 30.3) and estrogen-negative feedback starts to operate in females.[289,297] The fact that the arrival of the ARH fibers in the preoptic region could contribute to the maturation of estrogen negative feedback is strengthened by a recent study showing that perturbing the establishment of these ARH projections during the infantile period blunts the LH response to ovariectomy in juvenile mice, i.e., that the ability of the GnRH neuronal network to increase its secretory output in response to the loss of inhibitory control by estrogen is disturbed in these animals.[417] Efferents from other nuclei of the tuberal region of the hypothalamus that are functionally connected with GnRH neurons,[98,100] such as the VMH and DMH, also develop postnatally but are seen to reach the preoptic region earlier than ARH fibers.[416] Intriguingly, analogous to the critical period during neonatal life when sex steroids may specify sexually dimorphic patterns of development in the mammalian forebrain,[382,418,419] it has been shown over the past decade that the establishment of at least some of the projections of ARH neurons (e.g., the projections of the POMC and NPY/AgRP neurons) is also determined by metabolic hormones (e.g., leptin, ghrelin, and insulin) during a second critical period of postnatal development.[390] This developmental activity of peripheral metabolic signals is restricted to a neonatal/infantile window of maximum sensitivity characterized by the sequential elevation of the secretion of leptin,[420,421] which promotes ARH axon growth,[422,423] and of ghrelin,[424,425] which arrests this growth,[426] while insulin levels, which reflect the metabolic state of the pup, fine-tune these events.[427]

Synapse formation. The formation of synapses is a dynamic process that follows the development of axonal projections, and appears to continue well past the third week of postnatal life in the hypothalamus. For example, Reier and collaborators have reported that synapses in the preoptic region, which are sparse at birth, are primarily formed during the first 10–15 days of postnatal life and reach adult levels by P25 in rats of

both genders.[428] These ultrastructural changes are paralleled by an increase in the number of dendritic spines, as visualized by light microscopy in Golgi–Kopsch preparations,[428] which are thought to represent the location of predominantly glutamatergic excitatory inputs to these neurons.[429] As mentioned in the former section, GnRH neuronal somata and dendrites are themselves subject to an increase in spine density during postnatal development.[358,359,361] As in the preoptic region, in general, these changes occur between P10 and adulthood[361] and are concomitant with the arrival of neuronal projections from the ARH.[416] Promising preliminary studies from our laboratory show in addition that these events, which occur during the infantile period, are accompanied by a wave of astrogliogenesis.[430] Since astrocytes have been shown to be required for synaptogenesis,[431,432] and since altering astrocyte signaling[259] or preventing local cell proliferation around GnRH neurons during the second postnatal week by the stereotaxic injection of beads that release paclitaxel, an inhibitor of mitosis[430] leads to delayed puberty, the birth of new astrocytes during the infantile period may play an important role in this wiring process and could constitute one of the signals that set in motion the initial gonad-independent central activation of the gonadal axis.

Components

TRANSSYNAPTIC INPUTS

Glutamate. In accordance with the aforementioned neuroanatomical data, hypothalamic glutamate content gradually increases during postnatal development to reach a maximum after the onset of puberty.[433] In vivo push–pull perfusion experiments show that glutamate release from the preoptic region of female rats increases during the juvenile–peripubertal periods,[434] and is markedly elevated during the LH surge induced by gonadal steroids in ovariectomized animals.[435,436] Concomitantly, the ability of glutamate receptor agonists to stimulate GnRH release from hypothalamic explants increases gradually during the infantile–juvenile periods,[437] a phenomenon that is accompanied by an gonad-independent increase in NMDA-type glutamate receptor activity.[438] In vivo, gonadotropin release can be induced in sexually immature rats by treatment with the glutamate receptor agonists kainate and NMDA.[439–442] Remarkably, NMDA treatment in P16 infantile rats causes a significant increase in FSH release,[442] and the administration of NMDA advances the timing of puberty in rats[441,443] whereas treatment with NMDA antagonists delays it.[444–448] Together, these findings strongly suggest that the gradual increase in glutamatergic inputs to the preoptic region is a physiological component of central mechanisms underlying both the infantile activation of the gonadal axis and the first preovulatory surge of gonadotropins.[442,444–448]

The source of the glutamatergic inputs that mediate these changes in glutamate transmission during postnatal development remains to be determined. Several hypothalamic areas projecting to the preoptic region, such as the ARH, VMH, DMH, and PMv, and the preoptic region itself expresses type 2 vesicular transporters, an anatomical marker of glutamatergic cells.[101,449–452]

The investigation of glutamate receptor expression in GnRH neurons shows that up to 50% of them express the NMDA glutamate receptor[453–455] or the GluK5 kainate receptor (KA2).[456] The expression profile of the obligatory NR1 subunit of the NMDA glutamate receptor markedly increases in GnRH neurons during the infantile and peripubertal periods in mice[455] and rats,[454] respectively. In addition, electrophysiological studies have demonstrated that GnRH neurons also express functional AMPA receptors.[457,458] Even though GnRH neurons receive glutamatergic connections from diverse brain regions, including the AVPV, the electrical stimulation of which evokes monosynaptic NMDA and AMPA currents in GnRH neurons,[459] surprisingly, the deletion of neither AMPA nor NMDA glutamate receptors in GnRH neurons alters the onset of puberty or fertility in mice of either sex.[460] This study suggests that the expression of neither of these receptors in GnRH neurons is essential for the onset of puberty, and that, if they are important, their loss can be compensated for by the remaining glutamate receptor or by other afferent inputs of either neuronal or glial origin (see paragraphs below detailing nonclassic inputs).

GABA. γ-Aminobutyric acid (GABA) is a dominant neurotransmitter in the hypothalamus[461] and is synthesized from glutamate via a decarboxylase reaction catalyzed by two forms of the enzyme glutamic acid decarboxylase (GAD), GAD-65, and GAD-67. Expression levels of GAD mRNA increase in the preoptic region initially during the neonatal/infantile period[462] and then again during juvenile development.[463] GABA concentrations in brain tissues follow the same temporal pattern.[433,464,465] However, push–pull perfusion experiments suggest that GABA release from the preoptic region decreases during the juvenile–peripubertal transition period.[434]

Although the global effect of GABA on the onset of puberty and the GnRH network appears to be inhibitory,[466] emerging evidence suggest that its direct effect on GnRH neurons could be excitatory.[467] The infusion of GABA or the $GABA_A$ receptor agonist muscimol into the third ventricle or the preoptic region blocks the preovulatory surge of LH in intact female rats[468,469] and reduces the size of the estrogen-induced LH surge in ovariectomized rats.[470] In contrast, both intraventricular and intravenous infusions of bicuculline, a $GABA_A$ receptor blocker, advance the timing of the LH surge in proestrus and in gonadal-steroid-primed ovariectomized

rats.[471,472] Most GnRH neurons express functional ionotropic $GABA_A$ receptors that are synaptically activated both in brain slice preparations[457,459,473–476] and in vivo.[477] The various methods used to study GABA receptor expression in GnRH neurons clearly show that these neuroendocrine neurons express several different $GABA_A$ receptor subunits,[478–482] as well as the $GABA_B$ receptor.[483] Although GnRH neurons appear to express $GABA_A$ receptors throughout development, the subunit composition of these receptors changes considerably over time,[478,484] with a decrease in the range of subunits expressed before the end of the infantile period.[478] Both naturally occurring and induced $GABA_A$ receptor activation in GnRH neurons appear to be excitatory in nature throughout life;[459,485–487] however, as for the AMPA and NMDA glutamate receptors,[460] the knockdown of $GABA_A$ receptor signaling in GnRH neurons has no major effect on puberty onset or fertility.[488] This last study, in which the GABA response of GnRH neurons was reduced by 70–90%, suggests that either GnRH neurons compensate for their electrical excitability by other unknown inhibitory afferents or that normal levels of $GABA_A$ receptor-mediated transmission in the GnRH neuron are not important in shaping GnRH secretion at the median eminence.

Although changes in gonadotropin output triggered by experimental manipulations of the GABAergic system are contrary to those induced by the manipulation of the glutamatergic system, it is clear that both systems are tightly intertwined. Because glutamate is the natural precursor for GABA synthesis, any changes in glutamate availability directly affect GABA levels.[489] In addition, the two neurotransmitters have been shown to modulate each other's effects at both presynaptic and postsynaptic sites in the developing brain.[490] Unusually, AVPV neurons innervating GnRH neurons have been shown to co-express and co-release GABA and glutamate,[452,459] and the number of dual-transmitter-expressing neurons appears to increase around the onset of the LH surge and to be tightly regulated by estrogens.[452] One could hypothesize that such GABA/glutamate interactions also play an important role in the timely activation of the GnRH system during postnatal development.

Kisspeptin. During the last decade, kisspeptin,[491] a potent neuropeptidergic activator of GnRH neuronal activity, has emerged as a key player in the regulation of the onset of puberty and reproductive function.[492–495] Several comprehensive reviews on kisspeptin signaling and puberty have been published recently;[12,13,496–498] the following paragraph will therefore limit itself to the most salient aspects of the relationship of kisspeptin signaling with the timing of puberty.

Detailed studies in rodents have identified two prominent populations of kisspeptin neurons in the hypothalamus, one located in the ARH and the other in the AVPV, that are differentially regulated by gonadal steroids acting through ERα, and possibly the androgen receptor.[415,499,500] These populations are sexually dimorphic, with kisspeptin neurons being predominantly present in the ARH of both sexes,[492] where they have been proposed to mediate the negative feedback effect of sex steroids upon GnRH release,[499] however, they are present at a greater density in females than in males in the AVPV, where they are poised to elicit the preovulatory GnRH/LH surge through positive feedback mechanisms.[9] In addition, kisspeptin neurons of the ARH, which in contrast to those of the AVPV also express neurokinin B and dynorphin-A,[404–408] have recently been proposed to play a role in the control of pulsatile GnRH release via autaptic self-activation by neurokinin B and inhibition by dynorphin-A.[405,501,502] In both male and female rodents in adulthood, the expression of mRNA for the neurokinin B gene, *Tac3*, in arcuate neurons is inversely correlated to circulating levels of gonadal steroids, further supporting the putative involvement of these neurons in mediating the negative feedback of the gonads upon GnRH release.[408,503,504] The neurokinin B system in the ARH, which is sexually dimorphic,[404] has been shown to be immature at birth and to acquire adult features during postnatal development.[505] Surprisingly, rodents in which the activity of the neurokinin B receptor NK3R has been pharmacologically or genetically impaired[504,506,507] show normal puberty and only a mildly altered reproductive phenotype, unlike humans, in whom mutations disabling NK3R result in abnormal pubertal development and hypogonadotropic hypogonadism.[508] Whether the lack of effect of NK3R mutations on puberty reflects the ability of other tachykinin receptors to mediate NKB effects, as has been suggested in adults,[509] remains unclear. A recent report that the blockade of the kappa-opioid receptor advanced puberty in rats [502] points to a possible role for dynorphin in these neurons in pubertal development, although the deletion of this receptor decreased LH secretion in ovariectomized adults.[405]

Both mice and humans harboring deleterious mutations in the genes encoding kisspeptin (*Kiss1, KISS1*)[402,510,511] or its receptor (*Kiss1r, KISS1R*),[103,104,512–514] exhibit hypogonadotropic hypogonadism and infertility, whereas individuals carrying an activating mutation for *KISS1R*[515] show central precocious puberty. Even though spermatogenesis and ovulation appear to be severely impaired in most kisspeptin and KISS1R mutant mice, in some mouse lines, subsets of females undergo vaginal opening and show estrous cyclicity, whereas males exhibit varying degrees of spermatogenesis, ranging from arrest at meiosis II to the appearance of mature sperm.[511,516] The use of acyline, a GnRH receptor antagonist, has revealed that this remnant sexual activity, which can lead to estrogen-dependent increases in LH release in ovariectomized mutant mice,[512] and is also observed

in patients,[104,517] is mediated by hypothalamic GnRH signaling.[512,516] Importantly, an analysis of ovarian histology in mutant females exhibiting estrous cyclicity shows that these cycles are in fact anovulatory, since these ovaries contain follicles at all stages of development and a large number of atretic follicles, but no corpora lutea.[516] These findings, together with data showing that the proportion of GnRH neurons that respond directly to kisspeptin increases dramatically between the peripubertal period and adulthood,[493] and that kisspeptin-KISS1R signaling appears to be essential for the GnRH neuronal activation that initiates ovulation,[518,519] suggest that kisspeptin neurons, especially the population residing in the AVPV, might play a particularly important role during sexual maturation at the time of puberty onset. The relevance of peripubertal kisspeptin signaling for the acquisition of sexual maturity is reinforced by the observation that the pharmacological blockade of KISS1R during this period blunts the onset of puberty in female rats.[520] However, studies in which ERα has been selectively invalidated in kisspeptin neurons, leading to precocious puberty,[521] also suggest that kisspeptin neurons play a key role during the late infantile period when kisspeptin fibers from the ARH first reach the preoptic region.[417] These fibers likely mediate the negative feedback of estrogens onto GnRH neurons[521] that has been shown to be initiated around this time,[297] and thus tightly control the timely progression of female sexual maturation during postnatal development.

The direct apposition of kisspeptin fibers to GnRH neurons can first be visualized during the juvenile-peripubertal period; it attains maximal levels in sexually mature animals, with 12% and 40% of GnRH neurons displaying the apposition of kisspeptin fibers to their cell bodies in male and female mice, respectively.[522] The use of the transgenic *Kiss1r-LacZ* knock-in mouse model has shown that 50–70% of GnRH neurons express the *Kiss1r* gene, as visualized by X-gal histochemistry.[494,523,524] By rescuing fertility through the reintroduction of KISS1R into GnRH neurons in systemic *Kiss1r* knockout mice or by causing infertility with the selective deletion of *Kiss1r* expression in GnRH neurons, Herbison and colleagues have recently demonstrated that KISS1R expression in GnRH neurons is essential for the onset of puberty and reproduction in mice.[525]

POMC-derived peptides. The anorexigenic (appetite suppressing) proopiomelanocortin (POMC)-expressing neurons of the ARH[526] constitute one of the estrogen-sensitive afferents to the preoptic region[389] that can be seen synapsing onto GnRH neurons.[527] The loss of ERα in POMC neurons has recently been shown to blunt the ability of estrogens to mediate their negative feedback regulation of gonadotropin secretion and to cause subfertility in mice.[528] Among the POMC-derived neuropeptides, β-endorphin appears to be relatively ineffective in modulating GnRH neuronal firing,[529] which is in good agreement with studies that have found no evidence for μ-opioid receptor expression in GnRH neurons.[530,531] In contrast, α-melanocyte-stimulating hormone (α-MSH) has been shown to promote the firing of the majority of GnRH neurons in the preoptic region via the direct postsynaptic activation of melanocortin receptor 3 (MC3R) and MC4R, both in adult and peripubertal female mice.[529,532] Together, these results suggest that hypothalamic POMC neurons, which lie at the crossroads between the central control of energy homeostasis and reproduction, may play a significant role in postnatal sexual maturation.

NPY/AgRP. The orexigenic (appetite promoting) NPY/AgRP ARH neurons,[526] which, like the POMC neurons, constitute estrogen-responsive afferents to the preoptic region,[389] establish morphological and functional synapses with GnRH neurons.[388,529,533,534] The intracerebroventricular injection of an antiserum to NPY delays the age at puberty in female rats,[535] whereas a single intracerebroventricular injection of NPY advances vaginal opening and the first ovulation in these animals.[535] In rats, NPY hyperpolarizes GnRH neurons through the Y5 NPY receptor.[534] In mice, the activity of the majority of GnRH neurons is found to be modulated by NPY, which acts either through Y4 or Y1 to excite or inhibit GnRH neuronal activity, respectively.[529] Although there is evidence for the stimulatory effects of Y4 receptor activation on the HPG axis in rats,[536,537] the deletion of Y4 signaling restores the onset of puberty in the leptin-deficient *ob/ob* background without having any beneficial effect on body weight.[538] Similarly, deletion of the *npy* gene in ob/ob mice improves fertility but only partially restores metabolic functions.[539]

In rodents, NPY neurons in the ARH co-express AgRP,[401] an endogenous MC3R and MC4R antagonist[540,541] that has been variously shown to inhibit, promote,[529] or have no effect[532] on GnRH neuronal firing when bath-applied to brain slices, and to completely block depolarization promoted by the MC3/4R agonist melanotan.[532] In addition, ablation of the *Agrp* gene and heterozygosity for *Mc4r* have both been shown to restore puberty in female leptin-receptor-deficient *db/db* mice.[532]

Overall, estrogen-sensitive NPY/Agrp neurons are, like POMC neurons, in an ideal position to integrate a wide variety of metabolic signals such as leptin and ghrelin, which access the hypothalamus via the portal blood vessels and the tanycytic barrier,[109,110,542] and to coordinate both feeding and reproduction.

Other factors. Other factors such as the catecholamines, vasoactive intestinal peptide, neurotensin, and the opioids (other than dynorphin A, discussed above) have been suspected of being involved in the timely control of sexual maturation and the onset of puberty (see for review Refs 14,16).

ATYPICAL INPUTS

Nitric oxide. Originally described as the endothelial-derived relaxing factor,[543–545] nitric oxide is a highly

labile, gaseous messenger molecule that is generated as a product of the conversion of L-arginine to L-citrulline. The physicochemical properties of NO are such that it cannot be stored in vesicles following its synthesis, but instead diffuses across biological membranes to exert its effect. However, the ability of NO to act is delimited by its half-life and the proximity of NO-containing cells to their targets. In the brain, NO is typically considered to be a retrograde neurotransmitter (i.e., one that increases the release of other neurotransmitters, such as GABA and glutamate, from presynaptic sites); however, it also acts at postsynaptic sites (see for review Ref. 546).

Since the early 1990s, NO has been known to be a regulator of LH secretion (see for review Ref. 378). Indeed, several pharmacological studies have suggested that NO is a key modulator of GnRH secretion,[547,548] and a contributor to the onset of the preovulatory surge of GnRH/LH both in rats[549–553] and mice.[523] For instance, the intracerebroventricular injection of antisense oligonucleotides to nitric oxide synthase (NOS) has been shown to block the LH surge in steroid-primed ovariectomized rats.[552] In addition, the intracerebral infusion of N(G)-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor) into either the preoptic region or the median eminence in rats results in a marked disruption of estrous cyclicity,[510,554] and studies administering L-NAME (which crosses the BBB[555]) through the drinking water have shown that even the systemic inhibition of NOS delays the onset of puberty in female rats.[556] Surprisingly, the first targeted disruption of exon 2 of the neuronal (n) NOS gene did not markedly alter fertility in mutant mice;[557] however, these mice retained residual nNOS activity.[557] In contrast, the deletion of exon 6, which harbors the catalytic domain of nNOS, has been shown to cause hypogonadotropic hypogonadism and infertility in mutant mice.[558]

Although the global effect of NO on the onset of puberty and the GnRH network appear to be stimulatory,[549–553,558] electrophysiological data show that the direct effect of NO, whether endogenously produced by neurons or released by exogenous NO donors, is inhibitory on GnRH neuronal activity.[559] The mapping of the nNOS isoform within the hypothalamus demonstrates that GnRH perikarya in the OVLT/MEPO area are surrounded by nNOS-containing neurons, but do not themselves express nNOS.[523,559–562] Interestingly, preoptic nNOS neurons express all the key receptors known to be involved in the control of puberty onset and fertility, such as ERα,[563,564] NMDAR,[510,560] the μ-opioid receptor,[565,566] and the leptin receptor.[567] In addition, a recent study has demonstrated the abundant apposition of kisspeptin fibers to preoptic nNOS-containing neuronal cell bodies and the expression of KISS1R by these neurons, as well as the involvement of the estradiol→kisspeptin/KISS1R→nNOS signaling pathway in the estrogen-mediated negative feedback of GnRH/LH release.[523] The pharmacological activation of NMDAR→nNOS signaling, which is involved in the onset of the preovulatory GnRH/LH surge,[510,568] has also been shown to be able to promote LH release in KISS1R and kisspeptin null mice.[569]

In addition to mediating estrogen-dependent neural inputs, such as kisspeptidergic signals, the population of NO-synthesizing neurons in the OVLT/MEPO has also been shown to sense leptin,[570] thereby transmitting information regarding peripheral energy stores to GnRH neurons that do not express the leptin receptor.[571] Together, these data raise the provocative notion that nNOS-neurons of the OVLT/MEPO could be a vital cell population for the rapid integration and transmission of both gonadal and metabolic signals in the neuroendocrine brain, and thus play a role in the timing of puberty onset.

PGE_2. Prostaglandin E_2 (PGE_2) is one of a number of prostanoids synthesized from arachidonic acid, and is produced from membrane phospholipids by a phospholipase A_2. Arachidonic acid is converted to bioactive prostanoids by the cyclooxygenases (COX-1 and COX-2) and a class of terminal synthases (see for review Ref. 572). Several studies suggest that PGE_2 is mainly derived from the COX-2 pathway.[573–575] PGE_2 signaling is propagated by four G-protein-coupled receptors, EP1–EP4 (see for review Ref. 576).

The first indication that PGE_2 was involved in the process of GnRH secretion was provided by experiments showing that PGE_2 injected into the third ventricle of the rat brain induced the release of LH into the general circulation[577] and of GnRH into the pituitary portal blood vessels.[578,579] To bring about the activation of the GnRH axis, PGE_2 acts at two main hypothalamic sites: the preoptic-anterior hypothalamic region in which GnRH cell bodies reside, and the tuberal region of the hypothalamus, which contains the median eminence and GnRH-releasing neuroendocrine terminals.[580] The use of COX inhibitors, such as indomethacin, has provided further support for a physiological role of the prostaglandins in the control of GnRH release. In rats, the intraventricular or intrahypothalamic administration of indomethacin inhibits both pulsatile LH release and the LH discharge induced by ovarian steroids.[581] Other studies have demonstrated that the microinjection of either aspirin, a nonsteroidal COX inhibitor, or N-0164, an antagonist of prostaglandin and thromboxane, into the tuberal region of the rat hypothalamus results in the suppression of ovulation.[582,583] Finally, experiments conducted using hypothalamic explants in vitro have revealed that PGE_2 is an effective stimulator of GnRH release from median eminence nerve terminals.[584–586]

A sizable body of evidence also implicates PGE_2 as a physiological component of the GnRH system during postnatal development. For instance, PGE_2 can induce the release of GnRH long before puberty in both mice and

rats.[259,587] Biochemical analyses during the last phase of sexual maturation, i.e., at the first proestrus, have demonstrated that the capacity of the reproductive hypothalamus to metabolize arachidonic acid through the COX pathway leads to a specific increase in PGE_2 synthesis.[588] This effect appears to be estrogen-dependent, since it is mimicked by the treatment of juvenile animals (early postweaning period), with estradiol at doses capable of inducing a preovulatory surge of LH.[588] More recent studies have shown that an estradiol-induced increase in hypothalamic PGE_2 levels can be seen even during the neonatal period in male rats.[589] Additionally, experiments showing that estradiol treatment upregulates both COX-2 mRNA and protein synthesis in the hypothalamus of female rats during postnatal development[590] raise the possibility that estrogens may act on COX-2 expression to promote PGE_2 synthesis at puberty.

Glia: the main source of PGE_2 within the GnRH neurosecretory system. Although PGE_2 was initially postulated to be an intracellular messenger produced by the binding of neurotransmitters to receptors located on GnRH neurons and acting within these neurons,[591–593] this concept has been revisited following studies showing that the actions of PGE_2 on GnRH release are initiated by its binding directly to specific membrane receptors[576] expressed by GnRH neurons,[594] and the recognition that astrocytes represent a major source of PGE_2 in the brain[595–597] (Figure 30.5). Two decades ago, seminal studies by Ojeda and colleagues revealed that the PGE_2-mediated activation of GnRH neuronal secretory activity, which is triggered by estrogen at the time of puberty, required the activation of growth-factor-dependent glial signaling pathways involving receptor tyrosine kinases of the ERBB family.[608–610]

Of the four known members of the ERBB family, three (ERBB1, ERBB3, and ERBB4) bind and are activated by cognate ligands. In contrast, erbB2 has no known ligand, and functions primarily as a modulator of the other members of the family.[611] Although erbB receptors do not appear to be expressed in GnRH neurons,[211,259,606,612] ERBB1, ERBB2, and ERBB4, but not ERBB3, are expressed in hypothalamic astrocytes, known to morphologically and physically interact with GnRH cell bodies[613–616] both in rodents and humans.[259,605,606] In addition, hypothalamic astrocytes express the erbB1 ligand, transforming growth factor alpha (TGFα), and several forms of the erbB4 ligand, neuregulin.[605,606,609,612] Importantly, gonadal steroids have been found to induce dramatic increases in the expression levels of the ERBB receptors and their ligands within the hypothalamus at puberty; no such changes are seen in the cortex or other brain regions unrelated to reproductive control.[606,609,612]

The pharmacological or genetic inhibition of ERBB1, ERBB2, and/or ERBB4 receptor signaling pathways delays the onset of puberty[259,609,617,618] and alters adult reproductive function in rodents.[618] In vitro studies using either hypothalamic explants or the coculture of primary hypothalamic astrocytes with a GnRH-producing neuronal cell line have shown that although erbB receptor ligands can stimulate GnRH release from the explants or neuronal cells, they do so indirectly, by inducing astrocytes to secrete PGE_2.[259,595,606,610,618] In addition, ligand-mediated activation of erbB receptors has been shown to promote morphological rearrangements in hypothalamic astrocytes[605] (Figure 30.5), raising the possibility that ERBB signaling may also influence the astrocytic coverage of GnRH neurons in vivo (see for review Ref. 376).

In vitro experiments suggest that ERBB signaling in hypothalamic astrocytes is functionally connected to the neuronal glutamatergic system, the primary mode of excitatory transsynaptic communication used by hypothalamic neurons,[619] and as discussed above, one that is known to increase GnRH secretion[620,621] and accelerate the initiation of puberty in both rodents and primates.[441,446,622] In hypothalamic and nonhypothalamic astrocytes alike,[596,623,624] transmitter spillover from nearby synaptic activity (i.e., synaptically released transmitters from synaptic terminals are believed to bind to cognate receptors located at the surface of glial processes enwrapping the synaptic bouton) results in an elevation of PGE_2 release (Figure 30.4).[607,625] For example, excess glutamate released by neurons can reach and engage biochemical signaling in astrocytes through the co-activation of AMPA and metabotropic glutamate receptors, leading to a ligand-dependent increase in astrocytic ERBB signaling and PGE_2 release[604] (Figure 30.5). This in turn signals back to GnRH neurons, facilitating neuroendocrine development and adult reproductive function,[259,618] and possibly explaining the lack of an effect of the deletion of specific glutamate receptors in GnRH neurons themselves on sexual maturation.[460]

PGE_2, a newly uncovered gliotransmitter within the GnRH neurosecretory system. Even though PGE_2 has been known to trigger GnRH release from hypothalamic neurons controlling reproduction for almost 40 years, it has only recently been identified as a potent excitatory regulator of GnRH neuronal activity, both in male and female mice.[603] Patch-clamp recordings in brain slices have shown that PGE_2 induces a reversible membrane depolarization of GnRH neurons, leading to the initiation of spike firing via a postsynaptic effect involving the activation of a nonselective cation current[603] reminiscent of the ones recently described in GnRH neurons by other groups.[626,627] Although GnRH neurons are known to express both the EP1 and EP2 subtypes of prostaglandin receptors in vivo,[594,628] the excitatory effect of PGE_2 on GnRH neuronal activity is selectively mimicked by the EP2 receptor agonist butaprost,[603] previously shown to promote GnRH release in the GnRH-producing neuronal

cell line GT1-7.[594] The PGE_2-mediated membrane depolarization of GnRH neurons has also been shown to require the cAMP/protein kinase A (PKA) pathway,[603] which is known to be coupled to the EP2 receptor[574,576] and to underlie the stimulatory effect of PGE_2 on GnRH secretion.[629]

As alluded to above, the selective disruption of erbB4 signaling in astrocytes by the overexpression of a dominant-negative erbB4 receptor, under the control of the human GFAP promoter, leads to diminished PGE_2 release in response to ligand-dependent ERBB4 activation, leading in turn to reduced GnRH release, delayed puberty, and disrupted adult reproductive function.[259,618] What is more, electrophysiological analyses have shown that the spontaneous activity of GnRH neurons in these animals is also decreased, and that this deficiency can be mimicked by the bath application of either fluoroacetate, an inhibitor of astrocyte metabolism,[630,631] or the COX blocker indomethacin, to slices of the preoptic region of wild-type animals.[603] The fact that GnRH neuronal

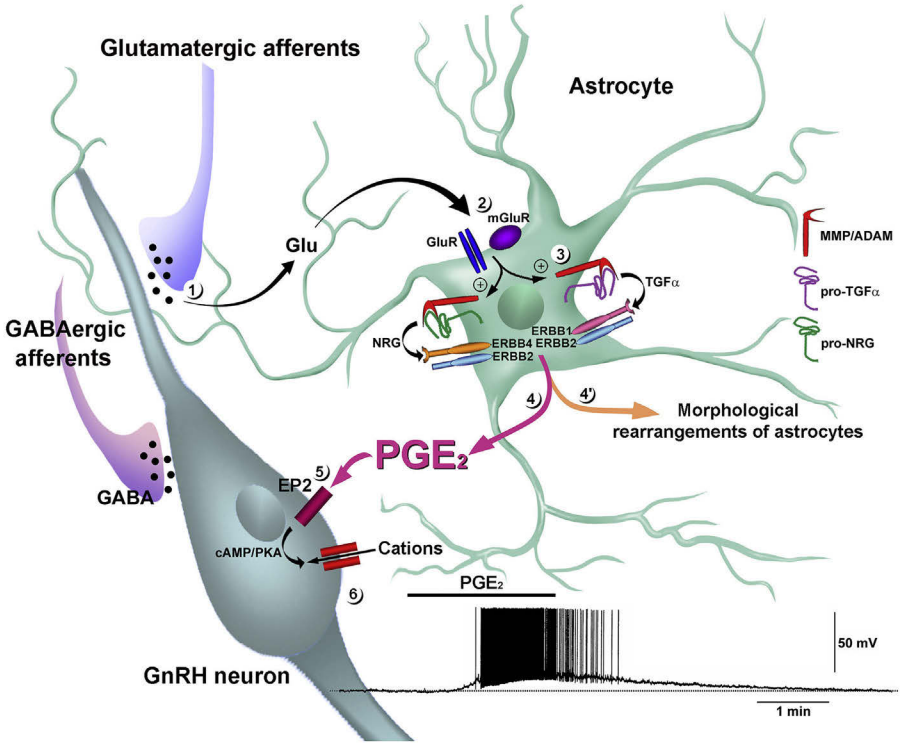

FIGURE 30.5 There is now a growing body of evidence to indicate that, concurrently with the transsynaptic regulatory mechanisms illustrated in Figure 30.4, cell–cell interactions involving nonneuronal cells such as astrocytes are also of critical importance for the regulation of GnRH secretion. Indeed, in the hypothalamus, as in most areas of the central nervous system, the classic one-way dialogue at the chemical synapse that forms the functional unit for the transmission of information between a nerve terminal and its target is being reevaluated on the strength of recent demonstrations that glial cells, the presumed electrically silent cohabitants of the nervous system, might be a critical third element of the synapse.[431,432,598–602] The present schematic diagram depicts one example of a mechanism by which hypothalamic astrocytes may contribute to the maturation of the GnRH neural network during postnatal development via the release of a gliotransmitter, prostaglandin E_2 (PGE_2), that not only potently stimulates the electrical activity of GnRH neurons,[603] but also modulates spine density in hypothalamic neurons.[590] Experimental studies have suggested that neuronally released glutamate (Glu) (1) coactivates metabotropic glutamatergic (mGluR) and AMPA glutamatergic receptors (GluR) in astrocytes, (2) stimulating the activity of zinc-dependent matrix metalloproteinases (MMPs) of the ADAM (a disintegrin and metalloproteinase) family, and (3) the MMPs catalyze ectodomain shedding by the pro-EGF ligands pro-TGFα and pro-NRG (proneuregulin). In particular, the processing of pro-TGFα has been shown to involve the metalloproteinase ADAM17, also known as tumor necrosis factor α converting enzyme (TACE). The subsequently released mature TGFα and NRG activate ERBB1/ERBB2 and ERBB4/ERBB2 heterodimers, respectively.[604] The co-activation of glutamatergic receptors induces the recruitment of ERBB1, ERBB4, and their pro-ligands to the cell membrane, where multiprotein complexes form, as demonstrated by the direct physical association of glutamatergic and erbB receptors (not shown). The activation of ERBB receptors in hypothalamic astrocytes promotes profound morphological changes, including cytoplasmic retraction and the elongation and stellation of processes[605] (4′). The activation of ERBB receptors also promotes the release of PGE_2[595,604,606] (4), which stimulates a cAMP/protein kinase A (PKA) pathway in GnRH neurons through the mobilization of EP2 receptors (EP2-R)[603] (5). The activation of this signaling pathway induces a reversible membrane depolarization of GnRH neurons leading to the initiation of spike firing via a postsynaptic effect involving the activation of a nonselective cation current[603] (6). The perception of synaptically released glutamate, GABA, or both, [607] by astrocytes, may contribute to the increase in hypothalamic GnRH feed-forward signaling in developing mice and rats. Indeed, the selective alteration of only one of these signaling components in astrocytes, such as ERBB4, hampers spontaneous GnRH neuronal activity[603] and delays puberty onset.[259]

activity under all these conditions can be rescued by exogenous PGE_2[603] strongly indicates that glial PGE_2 is an important component of the homeostatic mechanism controlling GnRH neuronal excitability. The role of glia in the control of GnRH neuronal activity is further supported by a recent study demonstrating that glial prostaglandins may decrease the efficacy of GABAergic inputs to GnRH neurons in ovariectomized mice.[607] Repeated action-potential-like depolarizations of a GnRH neuron have been shown to cause a short-term reduction in the frequency of spontaneous GABAergic postsynaptic currents in the neuron, suggesting the presence of local circuit interactions between GnRH neurons and their GABAergic afferents.[607,632] It is important to note that in this local circuit, the activation of $GABA_A$ receptors exerts a depolarizing effect that can trigger action potential firing due to the elevated chloride levels maintained in adult GnRH neurons.[467,485,633] Consequently, this represents a negative feedback loop in which depolarized GnRH neurons reduce the activity of their own excitatory GABAergic afferents. In addition to being steroid-dependent and under the influence of both glutamatergic and endocannabinoid signaling mechanisms via the activation of presynaptic metabotropic glutamate receptors and cannabinoid CB1 receptors, respectively,[607,632] this local negative feedback loop also requires the action of glial-derived prostaglandins.[607] Indeed, the incubation of brain slices with indomethacin, the broad-spectrum prostaglandin receptor antagonist AH 6809, or fluorocitrate, which like fluoroacetate, is a specific blocker of astrocyte metabolism, prevents the depolarization-induced suppression of GABAergic transmission in GnRH neurons.[607] Since GABA exerts a depolarizing action in this local circuit, we could envisage that glial prostaglandins, by suppressing excitatory drive, would reduce GnRH neuronal activity. Estradiol could also differentially influence this local inhibitory feedback to exert its positive or negative feedback effects.[607] Thus, in addition to exerting a direct postsynaptic excitatory action on the cell body of GnRH neurons, prostaglandins released from astrocytes could also participate in mechanisms that regulate the activity of their GABAergic presynaptic inputs. In the GnRH system, thus, PGE_2 fulfils all the criteria that qualify a compound as a "gliotransmitter"[634]: (1) it is synthesized by astrocytes; (2) its regulated release is triggered by physiological stimuli; (3) it acutely activates the firing of GnRH neurons and modulates the activity of their GABAergic afferents; and (4) it plays a role in an important physiological function, i.e., the neuroendocrine control of the onset of puberty and fertility.

Semaphorins. Recent evidence suggests that the semaphorins, members of a family of secreted guidance molecules, continue to be expressed in the postnatal brain and to have important implications for neuronal plasticity and nervous system physiology.[635,636] Strikingly, as can be seen below, several semaphorins also appear to play key roles in the GnRH system, controlling both the migration of GnRH neurons during embryogenesis, the timely onset of puberty,[164,167–169] and the amplitude of GnRH/LH release in adulthood by regulating the direct access of GnRH neuronal terminals to pituitary-portal blood vessels.[169,637,638]

SEMA3A: An endothelial-cell-derived GnRH axon-outgrowth-promoting factor that regulates the onset of the preovulatory surge. Of the semaphorins, Sema3A, a gene newly discovered to be involved in Kallmann syndrome, that acts as a guidance factor during the migration of GnRH neurons during embryogenesis,[163,164] as well as exerts both repulsive and attractive effects on growing axons,[639–641] is also expressed in the terminal field of GnRH neurons in the postnatal brain.[637] In the postnatal brain, however, the expression of SEMA3A is restricted to the fenestrated endothelium of pituitary-portal capillaries in the median eminence, and is tightly regulated by estrogens.[637] GnRH neurons are known to undergo extensive axonal growth towards the vascular wall during critical time windows, such as at the onset of the preovulatory surge, when a peak in the release of GnRH has to occur to trigger ovulation.[376] A recent study has highlighted a new mechanism by which endothelial cells of the median eminence release the 65 kDa isoform of SEMA3A (p65-SEMA3A) with precise timing during the ovarian cycle, promoting the extension of GnRH axon terminals towards the vascular plexus during the preovulatory surge, an effect that requires the expression of functional neuropilin-1, the SEMA3A receptor, in GnRH neurons.[637] The immunoneutralization of SEMA3A or the genetic impairment of SEMA3A release by endothelial cells impedes the onset of the preovulatory GnRH/LH surge.[637] Because ovarian-cycle-regulated GnRH axonal elongation in the postnatal brain is likely to depend on the coordinated activity of many extracellular factors, endothelial p65-SEMA3A may function in concert with other secreted molecules, including NO, TGF-β1, BDNF, and VEGF, which are particularly enriched in the capillary zone of the median eminence,[109,554,642,643] and may influence axonal plasticity through the modulation of the endothelial expression of or responsiveness to semaphorins.[640,644–646]

SEMA7A: A tanycyte-derived factor regulating neuroglial plasticity at the GnRH neurovascular junction. SEMA7A is another gene that has recently been found to be mutated in both patients with anosmic and normosmic hypogonadotropic hypogonadism.[170] It acts as a guidance factor for the migration of GnRH neurons during embryogenesis[168,169] and is also expressed in the terminal field of GnRH neurons in the postnatal brain.[647] The postnatal expression of SEMA7A is restricted to tanycytes of the median eminence, and is tightly regulated

by progesterone.[638] SEMA7A has been shown to play a dual role, inducing the retraction of GnRH terminals via one of its receptors, Plexin C1, although promoting the ensheathment and occlusion of GnRH nerve terminals by tanycytic end-feet through its other receptor, β1-integrin.[638] The genetic invalidation of Plexin C1 in GnRH neurons or of β1-integrin in the tanycytes of mice induces dramatic neuronal and glial rearrangements in the median eminence, which are correlated with impaired fertility.[638]

Together, these studies demonstrate that several molecules that play a key role during GnRH system ontogenesis are brought back into play during postnatal development to control neurosecretion, and any postnatal defect in the underlying mechanisms is likely to alter sexual maturation in the affected individuals.

Transcriptional Repression Mechanisms in the Control of GnRH Release

As shown in Table 30.2, the expression of the GnRH gene is regulated by a number of different molecules and transcriptional pathways, many of which are fairly widespread. In addition, although some of these molecules play a clearly activatory or inhibitory role, others, such as ERβ, can do both, depending on the context. A number of genes involved in regulating either the development of the GnRH system or the initiation of puberty are organized into functional networks that work more or less as discrete, although interconnected, units (see for review Ref. 648) Many of these do not directly regulate the transcription of GnRH, but act indirectly, for instance, by inhibiting the preexisting repression exerted by another molecule. The latter category has grown considerably over the last few years, as the involvement of several new molecules in the transcriptional activation or derepression of GnRH or its upstream activators, such as kisspeptin, has been revealed (reviewed in Ref. 649), transferring to the genetic level the notion that GnRH secretion occurs in the absence of inhibitory inputs to these neurons (reviewed in Ref. 650). How does this derepression work? In the case of kisspeptin neurons in the ARH, which are not sexually dimorphic but whose activity is necessary for pulsatile GnRH release, Lomniczi et al.[651] have shown that *Kiss1* expression is normally repressed by a polycomb complex, itself consisting of several gene products including those of *Eed* and *Cbx7*. At puberty, the promoters of these two genes are methylated, decreasing their expression and their participation in the polycomb complex binding to the *Kiss1* promoter and inhibiting transcription through other epigenetic modifications at the level of histone H3. The resulting derepression of *Kiss1* expression leads to the GnRH surge responsible for the pubertal activation of the pituitary–gonadal axis. On the other hand, steps aimed at stabilizing the polycomb complex and its binding and repression of the *Kiss1* promoter lead to the delay or nonoccurrence of puberty. Another gene involved in regulating GnRH release indirectly by transcriptional repression mechanisms is FGF21,[652] which acts by repressing vasopressin expression in suprachiasmatic nuclear afferents to kisspeptin neurons in the AVPV, thus inhibiting the kisspeptin-induced stimulation of GnRH neurons.[652] In addition, the fact that FGF21 is a metabolic signal secreted in response to temporary starvation provides another link between reproductive and metabolic states. The clinical relevance of such transcriptional repressive mechanisms to the control of reproductive maturation and function has been suggested recently by the finding that in a third of families in a cohort with central precocious puberty, there appears to be a loss-of-function mutation in the gene for the maternally imprinted makorin RING-finger protein 3 (*MKRN3*).[653] Although the precise function of this protein is not known, it belongs to the relatively conserved makorin protein family, and its expression in the ARH of mice is dramatically downregulated immediately before puberty.[653] Interestingly, MKRN3 is similar in structure to another RING finger protein, enhanced at puberty 1 (EAP1, also known as IRF2BPL), identified as being involved in controlling puberty onset both in rodents and primates.[654]

MODULATORY INFLUENCES REGULATING THE TIMING OF PUBERTY

The timing of the onset of puberty is also influenced by other factors, both intrinsic (e.g., hormones or signaling molecules secreted by other glands or produced during physiological processes) and extrinsic (environmental factors, both toxic and beneficial). An excellent and detailed account of several of these factors is provided in the previous version of this chapter by Ojeda and Skinner.[16] Here, I will limit myself to discussing one intrinsic factor (leptin) and one category of extrinsic factor (pheromones) respectively, for two different reasons. With respect to leptin, our knowledge of the involvement of this molecule in both metabolic and reproductive signaling has greatly increased over the last few years. Indeed, leptin appears to be a crucial link between the physiological systems regulating reproduction and metabolism, and the nature of the neuronal circuits mediating this cross talk are only just beginning to be unraveled. With respect to the pheromones, the fact that the principal rodent model for neuroendocrine studies until now has been the rat, in which pheromonal signaling plays only a cursory role, has led to an underestimation of their involvement in sexual maturation and function. However, pheromonal involvement in mice is

another matter entirely, and the advent of genetic mouse models for the study of reproduction makes it necessary to take into account their influence on, and interference with, the mechanisms being studied.

Leptin

As discussed in detail in Chapter 35, leptin, a peptide hormone mainly produced by adipocytes, plays a key role in the regulation of energy homeostasis and reproduction.[655–658] Although during the neonatal and infantile periods, leptin has no apparent effect on energy homeostasis,[659] after weaning, when pups have to seek their own food, leptin serves as an indicator of stored energy in the form of white adipose tissue,[660] and circulating concentrations of leptin are positively correlated with adiposity.[661] With respect to the onset of puberty, leptin appears to have two different but mutually overlapping functions, as a permissive signal that interacts with defined regions of the brain to allow or prevent the maturation of the HPG axis, and as an organizational cue that mediates the connectivity of key neuronal projections.

With regard to its permissive role in the onset of puberty, leptin deficiency, caused by a loss-of-function mutation of leptin or of its receptor, results in obesity and a failure to complete puberty, and consequently, to infertility, and these phenotypes can be rescued by treating individuals with leptin[662] or by reintroducing leptin receptor expression in neurons,[663] respectively. Conversely, the transgenic overexpression of leptin in mice causes precocious puberty.[664] Leptin does not appear to exert its effects on GnRH neurons directly, since the selective deletion of the leptin receptor in this neuronal population does not alter puberty onset or fertility,[571] but rather acts through cells afferent to these neurons,[101,665] through those that interact morphologically with them, or both.[666] Surprisingly, POMC and NPY/AgRP neurons of the ARH, which are critically involved in mediating leptin's anorexigenic effects,[667] do not seem to play major roles in the central action of leptin on the HPG axis, since the deletion of the leptin receptor in these neurons does not appear to induce any reproductive deficits.[668,669] Neither does the effect of leptin on puberty onset appear to involve kisspeptinergic ARH neurons,[449] of which only a very limited proportion express the leptin receptor.[666,670] The permissive action of leptin on sexual maturation could thus be mediated via neuronal populations that reside outside the ARH. An intriguing study by Elias and collaborators suggests that leptin-sensitive neurons that lie in the PMv could constitute one of these populations. Indeed, the selective bilateral lesion of the PMv prevents leptin-mediated rescue of fertility in ob/ob leptin-deficient mice without affecting the ability of leptin to restore body weight,[449] whereas the selective rescue of the leptin receptor in the PMv restores puberty onset and fertility in leptin-receptor-deficient mice without causing any change in body weight and food intake.[449]

Concerning its second function, leptin also appears to play a major role in the establishment of ARH projections to the preoptic region during the infantile period.[383] Indeed, in both rats and mice, there is a transient increase in circulating leptin concentrations during the first two postnatal weeks.[420,421,671,672] Bouret and collaborators have demonstrated that this rise in leptin promotes the outgrowth of ARH fibers by acting directly on ARH neurons expressing functional leptin receptors.[422,423] Although this surge in infantile leptin is independent of body fat mass, and the mechanisms of its regulation are still unknown, growing evidence indicates that the manipulation of neonatal feeding (i.e., either undernutrition or overnutrition) significantly alters circulating leptin concentrations during neonatal/infantile development.[672–674] This change in feeding has deleterious long-term effects on the animal's physiology, including alterations in the timing of puberty onset.[139,417,675] The importance of the organizational effects of leptin on reproductive circuits with respect to sexual maturation is highlighted by the fact that disrupting leptin-receptor-triggered STAT3 signaling causes a slight delay in puberty onset and altered fertility in mice.[676] This is particularly interesting when one considers that STAT3 signaling downstream of leptin has recently been shown to be required for the establishment of projections from the POMC neurons of the ARH,[423] which are not involved in mediating the direct effects of leptin on the activation of the HPG axis. However, further studies are required to determine whether infantile leptin promotes fiber outgrowth only in leptin-responsive ARH neurons or also in other ARH cell populations that send projections towards the preoptic region, such as the kisspeptin/neurokinin B neurons,[417] and whether these effects are mediated by direct or indirect trophic effects on the growing neurons themselves or by the triggering of chemorepulsive cues secreted by other leptin-sensitive neuronal populations that results in these projections leaving the ARH.

Pheromones

Pheromones are specific biologically active substances "which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process" as defined by Karlson and Lüscher in 1959.[677] In most mammals, including mice and rats, the nasal cavity contains at least two major sensory systems that may be involved in the detection of these chemical messenger molecules: the vomeronasal and the main olfactory

systems (see for review Refs 678,679). In rats, even though pheromones drive important behaviors such as the licking and cleaning of a pup's anogenital area, an act prompted by compounds that are produced by the pup's preputial glands and urine and required for the pups to defecate,[680] their effects on sexual maturation are relatively marginal.[681,682] However, this system plays a key role in sexual maturation and function in mice (see for review Ref. 683), whose interactions with their environment are largely triggered by pheromonal signals. Indeed, early studies have demonstrated a number of incontrovertible effects of primer pheromones in the latter species. For example, female mice living in groups modify or suppress their estrous cycle (the Lee–Boot effect),[684] whereas male urine can restore and synchronize the estrus cycle of noncycling females (the Whitten effect)[685] or accelerate puberty onset in immature females (the Vandenbergh effect).[686] Conversely, a very recent study has shown that the lachrymal glands of juvenile female mice secrete peptides in their tears that exert a powerful inhibitory effect on adult male mating behavior.[687] Besides, it has been known since the late 1950s that the exposure of a recently mated female mouse to a strange male prevents the implantation of the fertilized ova (the Bruce effect),[688] implying that the stud or its individual odor is memorized at the moment of mating and can be recognized later. These pheromonal effects, most of which are likely conveyed by stimuli present in the urine and acting via the vomeronasal organs, are powerful tools with which to probe the natural degree of maturation reached by the GnRH system during postnatal development (the Vandenbergh effect) and its function in adolescent mice (the Whitten effect), respectively. Indeed, studies by Bronson and colleagues have shown that exposing juvenile female mice between 26 and 30 days of age and weighing more than 17 g to a sexually mature male causes LH and FSH levels to peak, attaining preovulatory values, after 62 and 72 h, respectively.[689] Females that attain puberty at such a remarkably early age are capable of conceiving and carrying litters to term.[130] Interestingly, the immediate responses of the young females to male cues include a rapid release of LH, reminiscent of the LH minisurges in peripubertal female rats,[119] followed shortly afterward by a dramatic increase in serum estradiol.[689] Although the cohabitation of a single juvenile female with an adult male rapidly leads to pubertal ovulation,[126,690] young females housed in the absence of these cues show, after vaginal opening, greatly prolonged vaginal and uterine cycles that are anovulatory until quite an advanced age,[280] as elaborated in the section Rats and Mice as Model. In adolescent mice showing ovulatory cycles, the natural peak of LH release necessary for ovulation can be induced only by placing these mice on the day of diestrus I for 62 h in a cage that had previously held a sexually experienced male, i.e., by exposing them to male pheromones.[637,690] In striking contrast, rearing juvenile males in the presence of adult males depresses spermiogenesis and reduces plasma androgen concentrations,[691] an effect that could be linked to the significant decrease in LH and FSH concentrations in subordinate animals,[692] i.e., the inhibition of the hypothalamo-pituitary neuroendocrine system.

CONCLUSION

The study of the central control of puberty, or the attainment of sexual maturity, has advanced by leaps and bounds over the last few years. In contrast to our historical comprehension of this developmental phase as a fairly linear process, puberty now appears to be the culmination of an extraordinarily complex series of events occurring during embryonic and postnatal development, and ensuing from an intermingling of genetic, epigenetic, structural, neuroanatomical, and functional processes. The proper unfolding of this sequence of events requires a constant dialogue, initiated prenatally, between the hypothalamus and the periphery, mainly the pituitary and the gonads, but also other organs that transmit organizational and homeostatic signals to the brain and thus dictate the proper integration and function of GnRH neurons, the master regulators of the HPG axis. The elucidation of some of the cellular, molecular, and gene networks controlling every aspect of GnRH secretion has resulted in a multidimensional and ever-plastic picture, as befits a neuronal system controlling the most fundamental aspect of life—the propagation of the species.

In both males and females, GnRH neurons migrate from the nose to the brain during embryonic life under the control of a number of signals. At birth, the neuroendocrine terminals of these neurons target the pericapillary space of pituitary-portal blood vessels in the median eminence. GnRH neurons and the neural network to which they belong are then subjected to different periods of activation during postnatal development, leading to gonadotropin secretion. In females, the first activational period of the HPG axis coincides with the arrival of ARH fibers in the preoptic region (Figure 30.4), which results in an infantile surge of FSH that triggers the growth of the first pool of ovarian follicles, destined to ovulate at puberty, as well as the sporadic elevation of LH levels that contribute to their maturation (Figure 30.2). Low circulating estradiol levels produced by the growing follicles then gain progressive access to the hypothalamus, where they are posited to exert a negative-feedback effect on ARH neurons (Figure 30.4). Neurons and glial cells from the tuberal and preoptic regions of the hypothalamus, and possibly also neurons from other brain areas,

contribute to the maturation of the pattern of pulsatile GnRH secretion throughout the juvenile period, further promoting gonadotropin release and follicular growth. The second activational period occurs during the peripubertal period, when a diurnal rhythm of LH release that accentuates the functional development of the ovaries is established. A third and final activational period coincides with the moment when ovarian follicles reach full maturity, i.e., the Graafian stage, and release increasing amounts of ovarian steroids, specifically estradiol (Figure 30.2), which exerts a positive-feedback effect on the neurons of the AVPV (Figure 30.4), coordinating the onset of the first preovulatory GnRH and LH/FSH surge and thus triggering the first ovulation and conferring fertility on the individuals. In males, as in females, the primary events that initiate the onset of puberty originate within the hypothalamus. Following the neonatal masculinization of the brain via mechanisms involving the release of testosterone by Leydig cells that were born during embryogenesis, increases in FSH release (Figure 30.3) occur concomitantly with the arrival of ARH neuronal fibers into the preoptic region during the infantile period (Figure 30.4). This FSH then acts on the seminiferous tubules to promote the proliferation and differentiation of Sertoli cells, which facilitate spermatogenesis and enhance the function of Leydig cells born after birth. The latter, unlike embryonic Leydig cells, respond to LH by releasing testosterone, which plays a pivotal role in spermatogenesis. Between the juvenile and the peripubertal period, the frequency of secretion of both GnRH and LH increases gradually to attain an adult pattern. This maturation of the pattern of GnRH/LH release is paralleled by an extensive pruning of GnRH dendrites and changes in dendritic spine density, suggestive of an increase in glutamatergic afferents, and the appearance of the first motile sperm in the epididymis (Figure 30.3).

With so much that is known then, we come full circle to the question posed by the 125th anniversary edition of *Scienc*e, cited at the beginning of this chapter—What don't we know? The answer is, quite a lot. Even though in the process of deciphering these intricate pathways, several other aspects of the GnRH system have come to light in the last decade or so; for instance, the discovery that kisspeptin neurons are regulators of GnRH secretion, itself considered "the" master molecule for so long, many of the mechanisms underlying the cellular, genetic, and hormonal juggernaut leading to puberty are still obscure. For instance, even the very first step in the postnatal activation of the HPG axis, the arrival of afferents from the ARH, is steeped in mystery. How is the projection of these neurons to the preoptic area, where the majority of GnRH neurons reside, established, and what growth factors or guidance cues control this process? And more generally, what other neuronal populations influence GnRH neuronal activation, and how does this signaling take place? This is a particularly important question when one considers that, in our current state of knowledge, ARH neurons are poised at the interface between two primordial functions—reproduction and metabolic homeostasis. And perhaps the most fundamental question of all: how exactly is the expression of the GnRH gene itself controlled, and does this control play a role in the function of individual GnRH neurons and the activation of the HPG axis? The next decade should answer many of these questions, and raise new and even more interesting ones.

However, if the ultimate aim of these studies is to understand human reproduction, then it appears as if our understanding is fast approaching a plateau. All the mutations affecting the onset of puberty and fertility uncovered so far in human patients—whether with Kallman syndrome or various forms of hypogonadotropic hypogonadism or other manifestations of perturbations of the HPG axis—only serve to explain a third of the phenotypes seen in these patients. Although the exponential growth of screening methods at every possible molecular level will no doubt bring to light a few new molecular components and pathways, it seems equally possible that the actions of already identified genes and pathways could be altered in novel ways to result in the phenotypic permutations seen in humans. Several of these human disorders could actually be polygenic in nature, with "modifier genes" controlling the phenotype produced by a basic set of genes. Another candidate mechanism is the modification or suppression of naturally occurring transcriptional repression, whether by epigenetic modifications, as recently shown for *Kiss1*, the expression of miRNAs,[693] or other processes, remains to be determined. No doubt other mechanisms will be elucidated in the years to come. In this new and exciting "postgenetic" era of the study of the onset of puberty and the maintenance of reproductive competence, the use of rodent models, which can be easily manipulated and analyzed at every level from the addition of a simple methyl group to the production of young, will still have a place.

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CHAPTER

31

Puberty in the Sheep

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INTRODUCTION

Historical Overview

Historically, the sheep is preceded only by the dog in the temporal sequence of domestication. People of an agricultural, Mesolithic culture began the gradual domestication process of the wild Urial sheep (*Ovis orientalis*), probably in southwest Asia about 9000 BC; the Argali (*Ovis ammon*) and Mouflon (*Ovis musimon*) are now considered to have also contributed toward the Asiatic and European breeds of domestic sheep (*Ovis aries*) (see Ref. 1 for a review). The sheep, prized for its production of wool, meat, and milk, has been a successful species, adapting to divergent climatological conditions ranging from desert to Arctic areas and often living in marginal areas unsuitable for cultivation. Over 60 years ago, Hammond[2] published his classic study examining the effects of environment on puberty in lambs, pointing out that this "universal provider" was the most widely used domestic animal species for studies of female reproductive physiology. Subsequent studies over the next 25 years in sheep had little impact on the conceptual development of the field, but they were important from an agricultural standpoint. Understanding how puberty is timed is critical for developing practical strategies to synchronize lambing with market demand and for potentially decreasing the generation interval in farm animals, thereby increasing the overall production of food and fiber.

With the advent of radioimmunoassays that allowed detailed physiological studies on the control of gonadotropin secretion throughout puberty, the lamb developed into an important model in which to test theories based on work in other species because it has a large blood volume and a pubertal hiatus of 6–7 months that allowed for experimental manipulations at various ages prior to puberty. The first of these to be directly tested in the lamb was the "gonadostat" hypothesis which proposed that puberty resulted from a change in the hypothalamic set-point for estradiol negative feedback so that the low concentrations of this steroid in prepubertal animals could no longer hold gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) in check. This hypothesis—the origins of which may be traced back nearly half a century (see Refs 3 and 4 for a brief history)—was initially developed based on data from rats and was soon thereafter applied to humans. Ironically, at the same time that strong evidence supporting this hypothesis in female lambs was being developed in the late 1970s and early 1980s (described later in this chapter), work in female rats raised questions about its validity and data, first from rhesus monkeys and then humans, indicating that it was not applicable to much of the prepubertal period in these species. The current consensus appears to be that this hypothesis can account for the onset of puberty in sheep, ferrets, heifers, and male rats and for the final stages of reproductive development between menarche and first ovulation in primates.

With the fundamental neuroendocrine basis for puberty onset in sheep identified, workers turned to examining the control of puberty by photoperiod (in the 1980s) and nutrition. The former studies were relevant to many seasonally breeding species and the latter tested another long-standing hypothesis for the timing of puberty. Kennedy and Mitra[5] had hypothesized in 1963

Knobil and Neill's Physiology of Reproduction, Fourth Edition
http://dx.doi.org/10.1016/B978-0-12-397175-3.00031-4

that changes in energy availability serve as important cues timing puberty. They argued that sexual maturation is completed when sufficient somatic development has occurred so that excess energy reserves are available to support reproductive function. This hypothesis was soon applied to humans and has since been refined and championed by Rose Frisch and her colleagues. Work in prepubertal sheep (primarily in the 1990s) described in this chapter provided compelling evidence that suppression of somatic growth would dramatically delay puberty onset. Thus, the lamb provided a useful model to investigate how internal and external cues are channeled into the brain mechanisms that time the onset of fertility. During this same period, the technique for monitoring GnRH secretion in hypophyseal portal blood was applied to the lamb so that the relevant output of the brain could be directly monitored. This technique had been developed in adult sheep by Clarke and Cummins in Australia, refined by Caraty and Locatelli in France, and modified by Karsch in the United States to allow remote sampling (see review by Caraty et al.[6]).

Work in the late 1990s and early part of the twenty-first century focused on neural systems controlling GnRH secretion during puberty and the peripheral signals providing information about metabolic status. Because the pubertal increase in LH secretion (or the escape from estradiol negative feedback mentioned above) occurs much earlier in male than female lambs, interest also developed in sex differences for mechanisms timing puberty and how such differences develop prenatally. The long gestation of the sheep, which is approximately 5 months, coupled with the large size of the offspring permitted detailed studies of when and how fetal hormones exert their organizing action on the brain before birth to cause sex differences in the expression of pulsatile hormone secretion timing puberty. Despite the wealth of information available on pubertal mechanisms in lambs, progress in understanding these mechanisms in recent years has been limited. This lack of substantial progress is likely due to the loss of some more established investigators to retirement and a reduced entry of young investigators into the area, as well as the difficult funding environment of the day. In addition, while the sheep has many advantages regarding its use as an animal model, it is also a more costly animal to keep and maintain in comparison to smaller rodent models.

Because of the limited progress made in understanding the mechanisms underlying puberty onset in the sheep since 2006, much of the current text has not been changed from the previous version of this chapter. Specifically, it is organized into the same four major themes: (1) neuroendocrine sequence during the transition into adulthood; (2) internal determinants timing puberty; (3) external determinants timing puberty; and (4) sex differences in the timing of puberty. Nonetheless, updates in two areas are of particular note. First, consideration of the important role of a set of neurons in the hypothalamus called KNDy neurons—so named because of their coexpression of kisspeptin, neurokinin B, and dynorphin—to puberty onset is now included in the first theme. Second, we have expanded the consideration of the effect of factors such as androgens or environmental disruptors on development in utero and the subsequent effect on puberty in the fourth theme.

Comparative Considerations

From a broad vantage point, mechanisms underlying initiation of fertility in the sheep can be compared with those of other well-studied species to better understand the conservation and evolution of developmental strategies in differing ecologies. In this regard, the sheep is precocious in its development compared to the laboratory rat and rhesus monkey, both of which are altricial species. The lamb is well developed at birth, and many stages of maturation that are attained postnatally in species such as the rat (see Chapter 30) and primate (see Chapter 32) are achieved prenatally in the sheep. This rapid maturation is illustrated in Figure 31.1, which shows the relative growth curves for the sheep and for the human, another altricial species. Both are born at approximately the same body weight, but the lamb grows much more rapidly. Whereas by 30 weeks of age the weight of the human infant has only doubled, that of the lamb has increased tenfold and reproductive cycles have begun. Therefore, the degree of sexual development attained by the lamb in slightly greater than 6 months requires 13–15 years in the human child. Thus, the compressed developmental

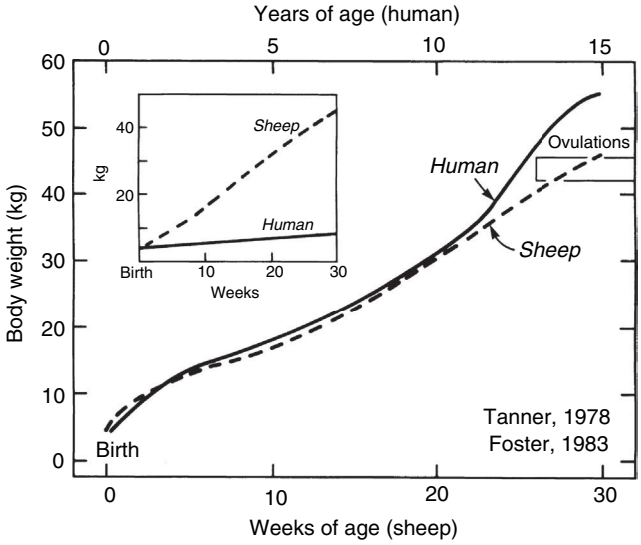

FIGURE 31.1 Growth based upon body weight from birth through the initiation of ovulation for sheep (mean) and human beings (50th percentile). Note the different *x*-axes. Growth during the first 30 weeks is shown (*inset*). *From Ref. 7, with permission.*

period of the lamb facilitates studies of sexual maturation of large, long-lived mammalian females.

Because its manifestation of puberty is subject to modification by external factors, the sheep is representative of many mammalian species living in natural conditions. Most mammals reproduce seasonally; as a consequence, their sexual development is influenced by environmental changes (food availability, photoperiod, temperature, rainfall, etc.). Which of these cues predominates depends on several factors, including the physical size of the species (larger animals have lower metabolic rates and more capacity for energy storage), longevity, and seasonal variation in local environment (see Chapter 34). The modern human deviates from the generalization that breeding in mammals is seasonal. Because of this, it could be argued that the expression of sexual maturity in most mammals is more complex than that of the human. As for all species, the young of seasonal breeders must grow to become sexually mature. However, they must also coordinate the timing of puberty with environmental changes such that their first matings occur at the same time as sexual activity is in progress in adults during the annual breeding season.

Definitions of Puberty in Female and Male Sheep

Puberty can be considered to be the process whereby an individual gains the capacity to reproduce. From a clinical or research standpoint, however, it is usually desired to provide a time point at which puberty occurs in order to classify its onset as precocious or delayed or provide a means to compare experimental groups. In the female lamb, the first mating can result in a pregnancy; therefore, many investigators use first estrus as the definition of puberty in the sheep. Others equate the initiation of ovulation with puberty. Because estrous behavior in sheep requires progesterone priming of neural substrates so that they can produce a behavioral response to estrogens (see Chapter 27), the first ovulation in lambs is not accompanied by estrous behavior. Indeed, two such "silent ovulations" may occur during puberty in lambs that have a short luteal phase after their first ovulation. Nevertheless, because first ovulation and first estrus typically occur within 2–3 weeks of each other in the sheep, a distinction between the two to define the onset of reproductive cycles normally has little practical usefulness, considering that the interval between birth and either of these events is 6 months or more. Puberty in males is a bit more difficult to determine as production of sperm, often used as an endpoint for designating achievement of puberty, is the summation of a process that begins approximately 60 days earlier. In addition, estrous behavior associated with puberty in the female can be detected through the use of vasectomized rams or by assessing the changes in progesterone secretion associated with luteal activity that can be measured by radioiummonoassay. In males, determination of production of mature sperm requires the collection and assessment of semen, a more complicated and laborious process. As will be noted later in this chapter, other definitions of puberty are applicable to reproductive development. For example, "neuroendocrine puberty" refers to maturation of the brain drive that produces hormonal signals to the gonads. This definition will be used in studies of steroid negative feedback control, as increased GnRH/LH secretion in response to reduced inhibition by estradiol during pubertal development is a common and critical event in puberty onset for both sexes. As such, this measure makes it easier to directly compare timing of puberty in the two sexes. In addition, because the discussion encompasses the broad aspects of seasonal influences on the timing of first ovulation and estrus, differences between "onset of breeding season" and puberty are considered. The reader is encouraged to deliberate the nuances of these related terms and concepts that apply to the transition to adulthood in the seasonally breeding sheep.

NEUROENDOCRINE SEQUENCE DURING THE TRANSITION INTO ADULTHOOD

Onset of High-Frequency GnRH Secretion Initiates the Transition into Adulthood

Because ovulation requires a surge of LH and follicle-stimulating hormone (FSH), any hypothesis for puberty in the female requires an explanation for how the preovulatory surge mechanism becomes operational. The hormonal events that lead to the preovulatory gonadotropin surge during the follicular phase of the adult estrous cycle have been particularly well characterized for the sheep (see Chapter 27). Briefly, during the 48- to 72-h follicular phase, GnRH pulse frequency becomes more rapid, and a sustained rise in basal LH secretion occurs as the frequency of LH pulses increases to greater than one per hour. The increased LH stimulus together with continued secretion of FSH drives one or more ovarian follicles to the preovulatory stage; estradiol production increases to high levels that are sufficient to activate the gonadotropin surge mechanism. Ovulation occurs, and one or more corpora lutea that secrete progesterone are formed.

In the immature female sheep, preovulatory gonadotropin surges do not occur spontaneously. Although the surge system is capable of function from a very early age, it remains dormant until puberty because of inadequate tonic (pulsatile) LH secretion. Pulses of LH, along with FSH, are ultimately responsible for the production of estradiol by the ovarian follicle. The pattern

of LH reflects that of its neurosecretotrophic stimulus, GnRH. In the prepubertal female, these LH pulses do occur; moreover, their amplitudes are as great or greater than those of the adult. The frequency, however, is much lower than that required to produce the large increases in follicle size and estrogen production required for triggering the GnRH/LH surge. The LH discharge rate in the immature female is no faster than once every 90–120 min for any prolonged period. During such slow frequencies, concentrations of circulating LH between each pulse return to low levels, and only transient rises in circulating estradiol are produced by small ovarian follicles. The young lamb is readily capable of generating requisite high-frequency GnRH/LH pulses, but this pattern is not produced because sensitivity to estradiol feedback inhibition is high. During puberty, a pronounced reduction in sensitivity to estradiol inhibition of GnRH secretion occurs, and the high-frequency rhythm of GnRH secretion is first expressed. As a result, the follicular phase begins and culminates in the first GnRH surge and ovulation. Subsequent events that occur during the pubertal transition, which include a short luteal phase in some lambs and first expression of sexual receptivity, will be considered at the end of this section, as will the switch from estradiol to progesterone as the primary regulator of LH pulse frequency with the emergence of corpora lutea.

The foregoing working hypothesis focuses on the mechanism governing the frequency of GnRH secretion, because this is the rate-limiting system in the onset of reproductive function. The rationale for this is based upon several considerations, namely: (1) the *potential* for the gonadotropin surge mechanism to respond to the stimulatory feedback action of estradiol exists long before puberty; (2) the *potential* for the ovary to produce follicular-phase levels of estradiol in response to hourly pulses of LH exists long before puberty; (3) the *potential* to produce high-frequency GnRH pulses exists long before puberty; (4) the response to estradiol inhibition of GnRH secretion decreases during the pubertal period. Evidence to support each of these contentions is presented in the following sections.

Competency of the Preovulatory Gonadotropin Surge Mechanism before Puberty

Numerous studies have demonstrated unambiguously that exogenous estradiol can induce an LH surge in the sexually immature female sheep.[8–15] As illustrated in Figure 31.2, the potential to respond to the stimulatory feedback action of estradiol is attained within a few weeks after birth. In the study shown, the amplitude of the LH surge increased progressively with age. By 12–20 weeks, the magnitudes of the induced LH surges were well within the range of those capable of producing ovulation. The sensitivity of the preovulatory

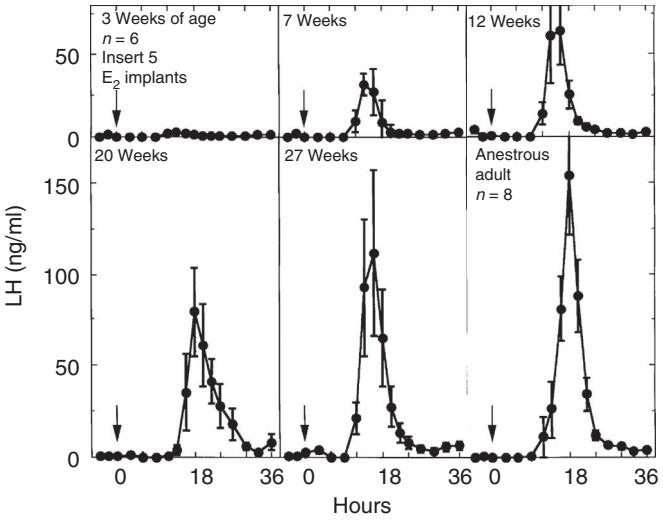

FIGURE 31.2 Development of the response to the stimulatory feedback action of estradiol on LH secretion in the female sheep. Estradiol was administered (*arrows*) subcutaneously by Silastic capsules for 96 h at each age in the same lambs. *From Ref. 10, with permission.*

gonadotropin surge system to stimulation has been tested in the prepubertal female several weeks before the first spontaneous ovulation.[12] Various lengths of Silastic capsules containing crystalline estradiol were used to produce physiologic rises of estradiol over a range of 2–10 pg/ml after endogenous estradiol had been removed by ovariectomy. The sensitivity in the immature female was found to be equal to that of the adult, and, remarkably, an estradiol increment as small as 2 pg/ml induced an LH surge. Moreover, administration of progesterone before estradiol in the immature female effectively prevented the estradiol-induced surge,[10] indicating that the potential for this blocking action becomes established well before its source of secretion, the corpus luteum. Taken together, these findings demonstrate that the mechanism governing the preovulatory surge system is exquisitely sensitive to the stimulatory feedback action of estradiol long before puberty, but that surges do not occur in the sexually immature lamb because they do not generate a sustained rise in endogenous estradiol. Although the ability to produce a GnRH/LH surge is obviously critical for puberty onset, very little if any attention has been devoted to understanding the neurobiology of surge development in the female aside from the role of androgens in masculinizing the surge system. This is likely due to the fact that, as discussed above, this aspect of the reproductive cycle is not "rate-limiting" to puberty onset.

Competency of the Ovary before Puberty

The ovary matures rapidly in the sheep. Antral follicles are evident before birth, but they do not respond to exogenous gonadotropins until after 2–4 weeks of

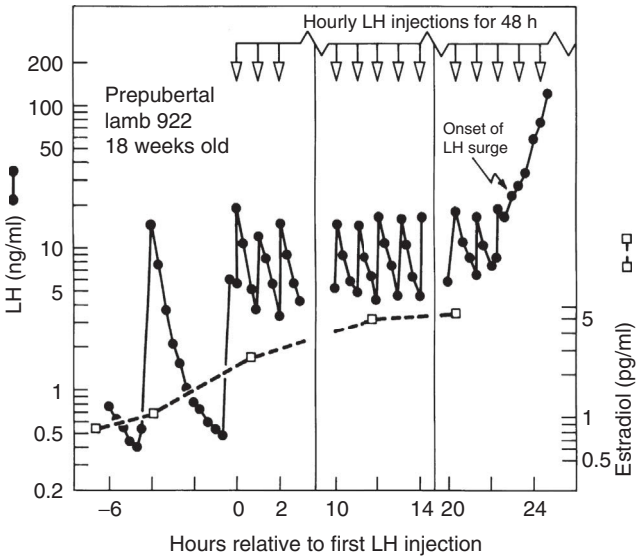

FIGURE 31.3 Rapid administration of LH induces ovulation in the immature sheep. Circulating LH and estradiol concentrations are shown at various times before and during hourly injections (*arrows*) of purified ovine LH in an individual female. High LH values between hours 22 and 24 reflect the initial portion of a preovulatory LH surge that was followed by an 11-day luteal phase with three corpora lutea. *From Ref. 25, with permission.*

age,[16,17] when granulosa and thecal layers become well developed.[18] At a slightly older age (5–6 weeks), exogenous gonadotropins (pregnant mare serum gonadotropin, human chorionic gonadotropin) can induce ovulation and formation of corpora lutea.[19] These exogenous gonadotropins can act by stimulating follicular growth and increasing estradiol production to activate the preovulatory gonadotropin surge mechanism,[20] much the same as occurs during the normal follicular phase (see Chapter 27). Moreover, the capacity of gonadotropin-primed follicles from 10- to 16-week-old lambs to secrete sex steroids in vitro was found to be similar to that of follicles from adults.[21] The precocious development of the lamb ovary has been used to practical advantage. Induction of ovulation by exogenous gonadotropins before the normal time of puberty[22,23] permits early mating to reduce the generation interval. Although, in principle, this could increase the overall lifetime agricultural production of ewes, fertility is low in such females as for young females of any species.

The competency of the lamb ovary to behave in an adult-like manner before puberty has been demonstrated in a more physiologic framework.[15,24] In the study shown in Figure 31.3, purified ovine LH was injected (intravenously) hourly for 48 h into prepubertal lambs to simulate a key aspect of the follicular phase of the estrous cycle—high-frequency LH pulses. The dose of LH used was chosen to replicate the amplitude of the pulses that are characteristic of the immature lamb (Figure 31.3, 6-h period before treatment). In response to hourly LH pulses, circulating estradiol rose to follicular phase concentrations and a preovulatory surge occurred, which resulted in ovulation and the formation of progesterone-secreting corpora lutea. Remarkably, for some prepubertal females in the study, as few as 22 hourly injections were required to initiate the sequence of ovulatory events. By contrast, high-amplitude, low-frequency pulses of exogenous LH are ineffectual in this regard.[15,24] Therefore, the absence of large, sustained estradiol rises in the prepubertal lamb is not due to immaturity of the ovary, but rather to the lack of a sustained, high-frequency gonadotropic stimulus.

Parenthetically, it should be mentioned that the time of spontaneous puberty and subsequent ovarian cyclicity is not advanced after the induction of ovulation in the immature female sheep by administration of gonadotropins. For example, prepubertal females in which an ovulatory cycle is induced by hourly LH injections fail to exhibit multiple, consecutive reproductive cycles after the corpora lutea of the induced ovulation regress.[15,24] Such lambs spontaneously initiate their first estrous cycles at the normal age (~30 weeks).[24] According to the working hypothesis, these immature females return to the anovulatory condition after luteal regression because the frequency of endogenous LH pulses remains low, and hence is insufficient to develop a preovulatory follicle.

Competency of the Pituitary to Secrete Gonadotropins before Puberty

The foregoing considerations about the ovary and surge mechanism lead to the conclusion that simple hypogonadotropism underlies the anovulatory condition of the immature sheep. More specifically, the deficiency appears to be in LH, rather than FSH secretion, because administration of LH *alone* induces the follicular-phase sequence (23, see Figure 31.3). A threefold to fourfold increase in LH pulse frequency has been reported by Huffman et al.[26] to occur gradually over the last week before first ovulation in the lamb (Figure 31.4). Before that period, the frequency is variable, although it is typically low, with the interval between pulses being 2–3 h or more.[26–30]

In contrast to LH, whether there is a role for FSH in the transition into adulthood in the sheep cannot easily be resolved. Circulating FSH in the lamb is well within the adult range by 10–12 weeks of age.[27,31] In one study, a subtle rise in peripheral FSH concentrations was detected 7–10 days prior to the first preovulatory surge in gonadotropins in samples of blood collected every 4 h.[32] However, in another study, this increase was not detected when circulating FSH was monitored more frequently (12-min intervals for 6 h) at various times before puberty[26] or at 2-week intervals during the first year of life.[33] Perhaps qualitative changes, rather than quantitative changes, in FSH may be important to the

transition into adulthood. An increase in the bioactive, or the more acidic, isoform of FSH was noted when puberty was induced by hourly GnRH administration.[34] No change in immunoreactive FSH was seen. However, a subsequent study[35] showed that the less acidic form of FSH was better at stimulating follicular development than the more acidic form. In addition, McNatty et al.[33] reported no change in either the bioactive or immunoactive forms of FSH with pubertal development; the more bioactive form was actually higher in lambs that did not reach puberty. Therefore, although FSH is required for follicular development and, thus, puberty onset, whether peripubertal changes in the secretion or bioactivity of FSH is involved is doubtful.

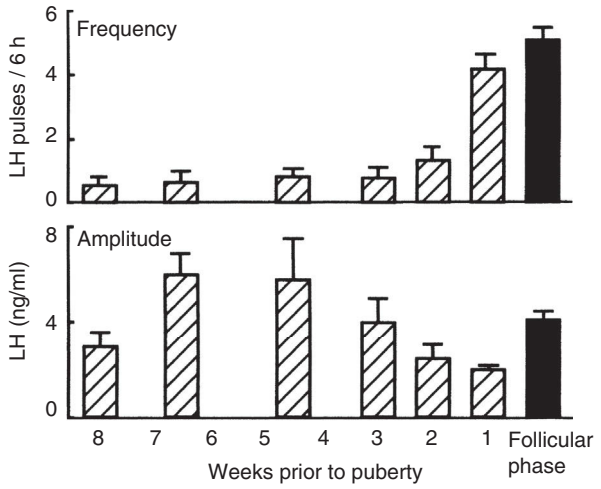

FIGURE 31.4 Mean (± SEM) LH pulse frequency (*top panel*) and amplitude (*bottom panel*) during the prepubertal period (*striped bars*) and the first postpubertal follicular phase (*solid bars*). Values for weeks 4–5 and 6–7 were combined to increase the number of observations contributing to the means. Each mean frequency was based on values from 7 or 8 lambs. Because of the absence of LH pulses during some collection periods, mean pulse amplitudes were based on values from 4 (weeks 3, 6–7, 8); 5 (weeks 2, 4–5); or 7 (week 1, follicular phase) lambs. *Redrawn from Ref. 26, with permission.*

At the outset, the simplest explanation for the low-frequency LH pulses before puberty is that the neuroendocrine system is not sufficiently developed to produce high-frequency GnRH pulses. This is not the case. Even during the early postnatal period, spontaneous hourly LH pulses can be readily produced under special conditions (removal of the ovaries, no negative feedback; Figure 31.5, bottom). Therefore, the potential to produce high-frequency LH (and GnRH) pulses, like those that initiate the follicular phase (Figure 31.3), exists from within a few weeks after birth in the sheep. Finally, the finding that high-frequency LH pulses can be produced before puberty (in the absence of steroid feedback) provides additional evidence that the pituitary is highly responsive to GnRH. Moreover, administration of GnRH readily produces LH pulses; if this is prolonged for 72 h[37] or more,[38] it can produce sufficient stimulation of the pituitary–ovarian axis to induce ovulation.

Competency of the Hypothalamus to Produce GnRH before Puberty

Determination of the pattern of GnRH to which the pituitary is exposed has been difficult for any species, largely because of the inaccessibility of the circulation between the hypothalamus and pituitary (portal vasculature) and because of the minute amounts of the neuropeptide actually secreted into the pituitary circulation. However, as described earlier, a technique to collect hypophyseal blood from unanesthetized adult sheep was developed in the 1980s. When this technique was adapted to younger animals and GnRH secretion was examined in the prepubertal lamb (19 weeks of age) without ovaries, the pattern of LH faithfully reflected

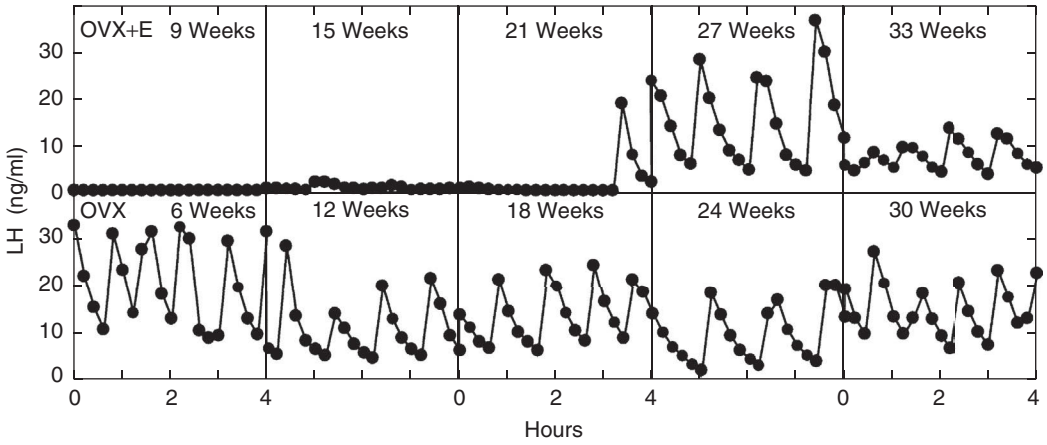

FIGURE 31.5 Patterns of circulating LH in the same ovariectomized (OVX) lamb in the presence (weeks 9, 15, 21, 27, 33) and absence (weeks 6, 12, 18, 24, 30) of exogenous estradiol (E, 2 pg/ml by implant). Samples were collected 3 weeks after insertion (*top*) or removal (*bottom*) of implant. Ovariectomy was at 3 weeks of age. *From Ref. 36, with permission.*

the pattern of GnRH secretion.[39] Each LH pulse was preceded by a GnRH pulse; moreover, each obvious GnRH pulse resulted in an LH pulse. Such findings provide the necessary direct evidence that the developing female sheep has the potential to produce high-frequency GnRH pulses long before puberty.

Decrease in Sensitivity to Estradiol Feedback Inhibition of GnRH Secretion during Puberty

The "gonadostat" hypothesis, which was originally developed based on data from rats, offers a conceptual explanation for why high-frequency GnRH pulses are not expressed in the immature female lamb. The prepubertal sheep seems to be very sensitive to estrogen-induced inhibition of GnRH/LH secretion; very low doses of estradiol (<1 pg/ml) delivered by subcutaneous (sc) implant will reduce the frequency of LH pulses to 50% within 24 h and a slightly greater dose (1–2 pg/ml) will completely suppress LH pulses.[40] Because of this highly suppressive effect of extremely low doses of estradiol in the ovariectomized prepubertal sheep model, it is of interest that the response of LH secretion to inhibition by a fixed, chronic low dose of estradiol decreases markedly during the pubertal period.[41] Although low physiologic concentrations of exogenous circulating estradiol were able to prevent high-level LH secretion in ovariectomized lambs before the typical age of puberty, the same estradiol levels were ineffective in this regard afterward (Figure 31.6). Circulating LH increased in these chronically estradiol-treated agonadal lambs when ovulations were beginning in ovarian-intact females. This leads to the conclusion that the decrease in feedback sensitivity to estradiol inhibition allows the follicular phase pattern of persistent high-frequency LH pulses to become expressed for the first time in the presence of the ovaries. This would rectify the typically hypogonadotropic condition of the developing lamb, and the ovarian follicle would be driven through the follicular phase of the first successful reproductive cycle—one that results in ovulation.

Detailed studies of the model used to examine changes in set-point to steroid negative feedback have revealed that estradiol administration (via Silastic implant) reduces LH pulse frequency, but not amplitude, in the immature ovariectomized lamb.[40,43] Accordingly, the subsequent increase in basal LH in such estradiol-treated females during the pubertal period reflects an increase in LH pulse frequency.[44] This phenomenon whereby high-frequency LH pulses become expressed during the pubertal period because of a reduction in sensitivity to estradiol inhibition can readily be demonstrated by periodically opening and closing the inhibitory feedback loop between estradiol and LH secretion within the same individual. For example, as shown in Figure 31.5, removal of the ovaries at 3 weeks of age results in high-frequency LH secretion by 6 weeks of age; insertion of an estradiol implant then suppresses LH secretion, as evidenced by the pattern at 9 weeks of age. Subsequent removal of the estradiol again reveals the potential for high-frequency LH secretion. This relationship was maintained upon insertion and removal of the estradiol implant with the same lamb until approximately 27 weeks of age, when insertion of the same estradiol implant had no apparent effect. This is about the age at first ovulation in intact lambs. Thus, before the age of puberty, estradiol is extremely effective in suppressing

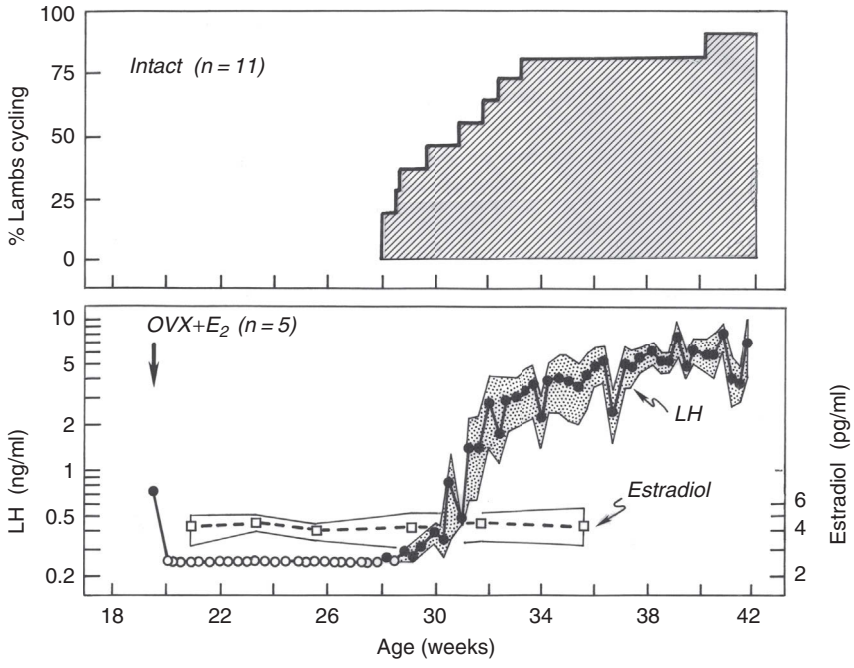

FIGURE 31.6 Response to estradiol inhibition of tonic LH secretion decreases during puberty. *Top*: Onset of reproductive cycles in untreated lambs based upon the appearance of luteal-phase concentrations of circulating progesterone. *Bottom*: Mean (± SEM) concentrations of circulating LH and estradiol (E_2) in ovariectomized lambs treated chronically with Silastic implants of estradiol from the time of ovariectomy (*arrow*); undetectable LH values (*open circles*). *From Ref. 42, with permission.*

pulsatile LH secretion, and it loses its effectiveness as the time of puberty is approached. Progesterone then assumes the role of the major inhibitory feedback control of GnRH secretion. Although the female sheep is responsive to the negative feedback action of progesterone on LH secretion before puberty,[45] this has no physiological relevance until the source of its secretion appears after first ovulation. When viewed in the context of the gonadostat hypothesis and pubertal process, these results offer additional strong evidence that in the female sheep, the mechanism governing GnRH secretion is highly responsive to estradiol inhibitory feedback before puberty, and that a decrease in response of the mechanism to estradiol feedback is responsible for the increase in GnRH pulse frequency that underlies the transition into adulthood.

Ultimately, the changing sensitivity to inhibitory steroid feedback control of GnRH secretion must be explained in electrophysiologic/neurobiochemical terms. In the adult of several species, there is strong inferential evidence for the changing electrophysiological activity of neurons that periodically secrete GnRH into the pituitary portal vasculature. LH pulses have been found to occur closely in time with volleys of electrical activity recorded from multiple neurons in the adult hypothalamus (sheep,[46] rat,[47] monkey,[48] goat[49]). A limited study has been performed in the developing sheep. In anesthetized male lambs, Pelletier and colleagues[50] recorded multiunit activity in an area between the optic chiasm and median eminence that was related to pulsatile release of LH in adult ovariectomized female sheep. Rhythmic changes in firing rates were recorded at 40–60 min intervals; however, because of the anesthesia, LH secretion was reduced such that pulses could not be detected. Hence, no correlation between electrical activity and LH secretion could be determined in this study.

In searching for a biochemical explanation for the observed decrease in sensitivity to estradiol during the pubertal period, Wilkinson and Landymore[51] have suggested that the opioid system may play a role. According to their hypothesis, endogenous opioids mediate inhibitory steroid feedback and a reduction in opioid inhibition provides a neurochemical explanation for resetting the gonadostat. Support for this idea was recently provided by the report that administration of a kappa-opiate receptor antagonist to female rats accelerated puberty onset.[52] This hypothesis has been investigated in the developing sheep by administering an inhibitor of endogenous opioids (naloxone) to female lambs at various ages.[53] Some were reared to undergo normal puberty (Figure 31.7, top panel, for representative females), whereas others were exposed to a photoperiod treatment that delayed puberty (Figure 31.7, bottom panels). In both groups, naloxone had no effect on LH secretion at the earliest age tested (9 weeks, data not shown); however, at all later developmental stages, the opioid antagonist increased LH pulse

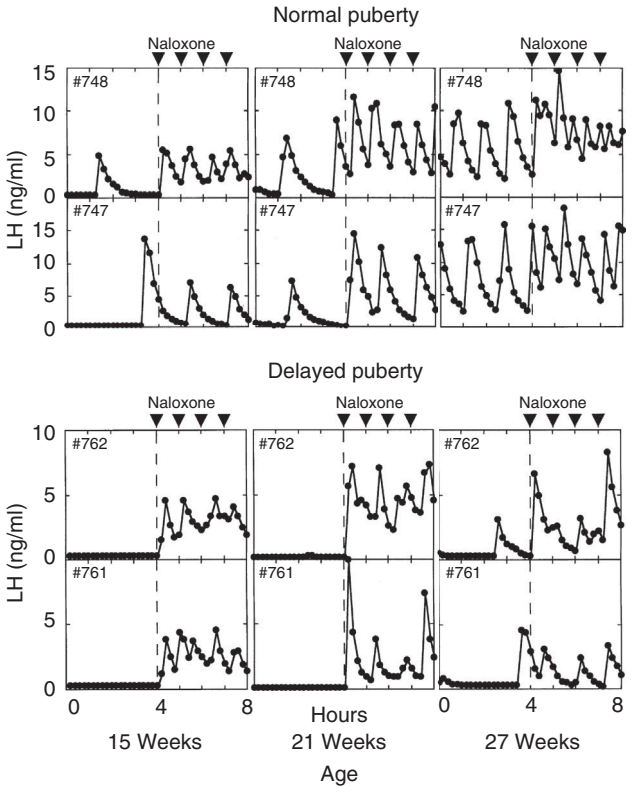

FIGURE 31.7 Effects of naloxone on serum LH concentrations in developing estradiol-treated OVX lambs at 15, 21, and 27 weeks of age. Data from four representative individuals are shown. During each naloxone test, the first 4 h served as a control period; four intravenous injections of naloxone (1 mg/kg body weight) were administered hourly for 4 h (*arrows*). *Top*: Two individuals (748 and 747) were raised on a permissive photoperiod; note the age-related increase in LH pulse frequency during the control periods. *Bottom*: Two individuals (762 and 761) were raised on an inhibitory photoperiod; note that LH secretion remains low in the control periods throughout the experiment. *Redrawn from Ref. 53, with permission.*

frequency. Importantly, the incremental change in LH pulse frequency after naloxone treatment did not differ with age. Even with photoperiodically delayed puberty, robust responses were induced after opioid receptor blockade. This leads to the conclusion that endogenous opioid mechanisms provide important inhibitory control of pulsatile LH secretion in the developing sheep. However, the ubiquity of response to opioid blockade in pre- and postpubertal females does not support the hypothesis that a reduction in opioid inhibition underlies puberty. A similar conclusion was reached in a comparative study of opioid blockade of LH secretion in developing male and female lambs; the response to naloxone administration is not sexually differentiated,[54] although the timing of puberty is.[55,56] This provides additional evidence that the response to naloxone develops very early in life and is not related to time of puberty. Finally, the basic premise that endogenous opioids mediate inhibitory estradiol feedback must be rejected because opioid

receptor blockade further increases LH pulse frequency in the lamb without ovaries.[53]

Studies in humans examining the association between gene mutations and hypogonadotropic hypogonadism have suggested an important role for two hypothalamic neuropeptides, kisspeptin and neurokinin B (NKB), in regulating GnRH release. Loss-of-function mutations in either kisspeptin[57] or its receptor, KISS1R (aka GRP54),[58,59] result in a failure to reach puberty. In addition, a mutation that apparently leads to prolonged intracellular activation by kisspeptin is associated with precocious puberty.[60] Administration of kisspeptin has been shown to stimulate LH release in several species, including mice,[61] rats,[62] nonhuman primates,[63] humans,[64] cattle[65] and sheep,[66] and this influence on LH secretion appears to be primarily due to actions on GnRH neurons.[61,62,66] Two populations of kisspeptin neurons are evident—one in the preoptic area/rostral periventricular area and one in the arcuate nucleus of the hypothalamus. In sheep, kisspeptin neurons in the preoptic area are generally believed to be involved in generation of the GnRH surge,[67] whereas those in the arcuate nucleus may regulate both tonic and surge release of GnRH.[68,69] A more detailed discussion of kisspeptin and NKB and their role in the adult sheep model can be found in Chapter 27. Few studies to date have examined the potential role of kisspeptin in timing puberty in the sheep, but those that have suggest that it could play a role in driving increased GnRH secretion. Hourly administration of kisspeptin in prepubertal female lambs elicits corresponding pulses of LH and eventually an LH surge,[70] and LH pulse frequency in ovariectomized lambs treated with estradiol during pubertal development is positively correlated with *Kiss1* mRNA-containing cell numbers in the middle portion of the arcuate nucleus.[71] Another study[72] found that kisspeptin-immunoreactive cell numbers in the arcuate nucleus are higher in postpubertal female lambs compared to prepubertal female lambs and that this increase is mirrored by an increase in the percentage of GnRH neurons receiving kisspeptin-positive close contacts identified at the level of the light/confocal microscope (Figure 31.8). In addition, that study also found that kisspeptin immunoreactive cell numbers increased following ovariectomy in prepubertal, but not postpubertal, lambs, indicating expression is inhibited by estradiol prepubertally. Kisspeptin or KISS1R deletion in the mouse[59,73–76] is supportive of an important role for kisspeptin regulating the control of GnRH secretion during sexual maturation in this species, but similar studies are not available for the developing sheep.

Similar, albeit more limited, information is available for NKB. Mutations in either the *TAC3* or *TACR3* genes, which encode for NKB and its receptor, respectively, are associated with hypogonadotropic hypogonadotropism in humans.[77] An important role for NKB or its receptor in rodent sexual maturation is somewhat unclear at this point as the effects of NKB on LH release have been reported to be stimulatory, inhibitory, or insignificant.[78–81] In addition, mice with a deletion of NK3R (an NKB receptor) appear to be fertile.[82] Recent work in prepubertal male nonhuman primates[83] and prepubertal female sheep[72] showed that senktide, an agonist for NK3R, stimulates LH release. Furthermore, although Nestor et al.[72] reported that NKB-positive cell numbers in the arcuate nucleus were not different between ovary-intact prepubertal and postpubertal female sheep, NKB fiber density within the arcuate nucleus was increased in postpubertal females compared to prepubertal females, perhaps indicating an increased synthesis and transport of NKB. In that study, NKB cell numbers did increase with removal of ovarian steroids at both stages, suggesting that NKB expression was under the control of estradiol-negative feedback.

Interestingly, kisspeptin and NKB, along with dynorphin, are frequently colocalized in the same neurons within the arcuate nucleus,[84] leading to the moniker of KNDy neurons. Although any role for dynorphin in the ability of sheep to express sexual maturity is largely unexplored, dynorphin has been shown to inhibit LH release and mediate progesterone negative feedback control of LH secretion in adult female sheep.[85–87] It has also been reported in adult female sheep that KNDy neurons express NK3R,[88] but not KISS1R,[89] whereas GnRH neurons express KISS1R, but not NK3R.[88,89] Along with evidence in rodents that kisspeptin neurons in the arcuate nucleus express kappa opiate receptors for dynorphin,[79] it has been postulated that NKB may act in a paracrine or autocrine fashion to stimulate kisspeptin release, which then acts to cause a pulse of GnRH to be released. This pulse is then terminated by local dynorphin actions on KNDy neurons. However, it should be noted that effects of NKB outside of the arcuate nucleus on LH release have also been reported.[90] Thus, although KNDy peptides appear to be necessary to express sexual maturity through the regulation of GnRH secretion, the exact mechanisms whereby they participate in this process remain to be determined. Finally, it must be emphasized that many neuropeptides could play an essential role in the regulation of GnRH secretion in addition to the KNDy peptides.

Workers using sheep, ferrets, and cattle as experimental models have embraced the gonadostat hypothesis as an explanation for the ultimate timing of the pubertal rise in GnRH/LH secretion (see Ref. 91 for a review). However, those working with the rhesus monkey, a subhuman primate, have differing perspectives (see Chapter 32). Studies[92,93] have been conducted testing the gonadostat hypothesis in the rhesus monkey using the same protocol as in the sheep (e.g., see Figure 31.6, bottom panel). The results reveal that low physiologic

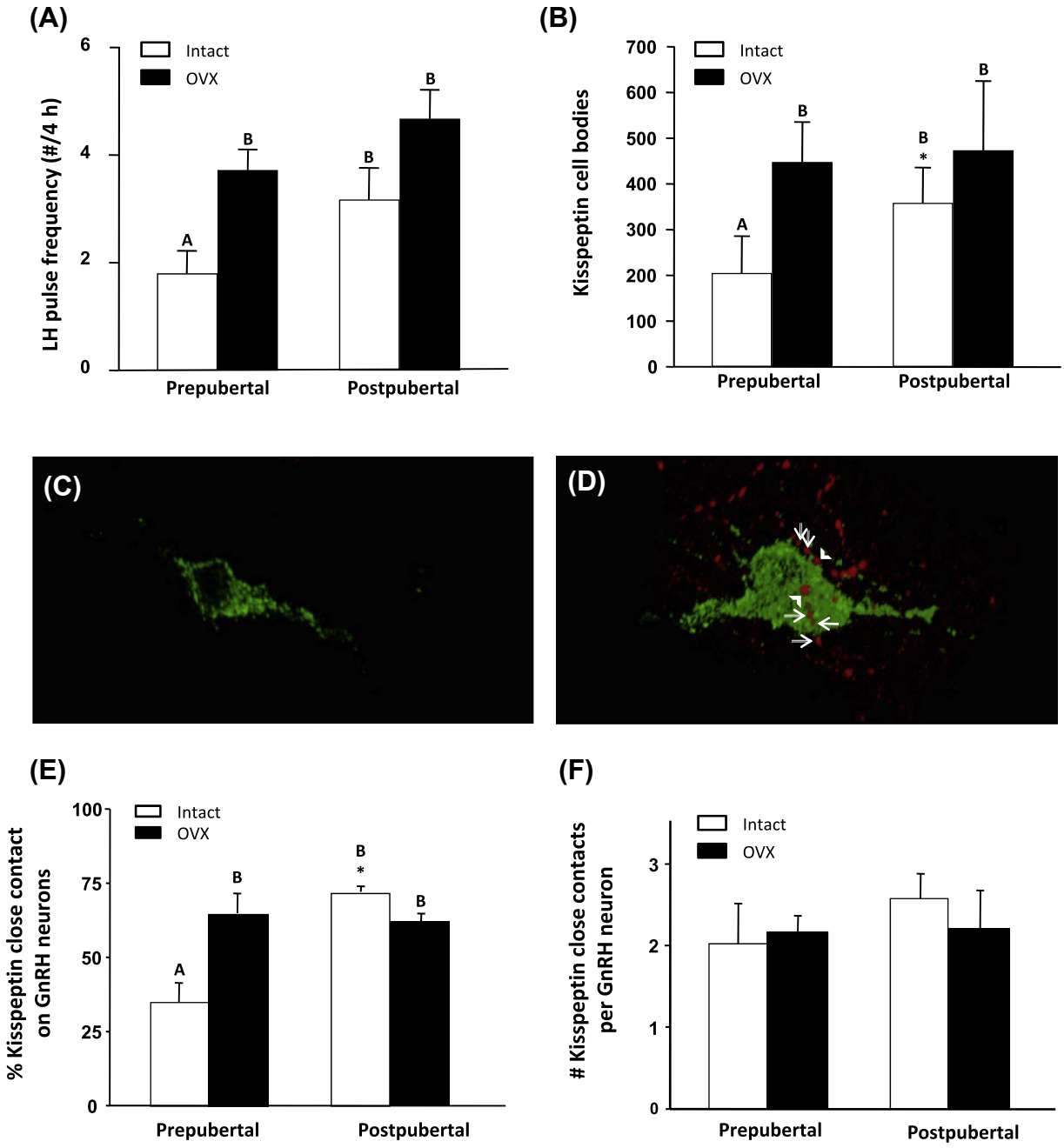

FIGURE 31.8 Changes in LH pulse frequency and kisspeptin during pubertal development. Panels C and D of this figure are reproduced in color in the color plate section. *Panel A*: LH pulse frequency in prepubertal and postpubertal female lambs that were either gonad-intact (Intact) or ovariectomized (OVX). Postpubertal intact lambs were in the early follicular phase. LH pulse frequency was higher in intact postpubertal female lambs than in intact prepubertal female lambs and rose following ovariectomy in the prepubertal, but not postpubertal, lambs. *Panel B*: Kisspeptin-immunopositive cell numbers were greater in postpubertal intact females than prepubertal intact females and increased with ovariectomy in the prepubertal, but not postpubertal, lambs. *Panels C and D*: GnRH neuron from a prepubertal female (panel C) and postpubertal female (panel D). Heavy arrows indicate point of contact between kisspeptin-containing varicosities and the GnRH neuron. Lighter arrows depict kisspeptin-positive nerve fibers. *Panels E and F*: The percentage of GnRH neurons exhibiting at least one kisspeptin close contact (panel E) was greater in intact postpubertal females than intact prepubertal females. This percentage increased with ovariectomy in the prepubertal, but not postpubertal, group. Numbers of contacts per GnRH neuron (panel F) did not differ among groups. *Redrawn from Ref. 72, with permission.*

concentrations of the steroid produce a potent inhibition of LH secretion for a prolonged period in the ovariectomized postmenarchial rhesus monkey; however, a rise in circulating LH eventually occurs; when it does, it is over the same age range as for first ovulation. Similar observations have been made by Wilson for the control of FSH secretion by estradiol in the developing rhesus monkey.[93] Thus, the sensitivity to estradiol negative

inhibition decreases in the postmenarchial monkey and is likely to be important for the first ovulation. Transient returns to periods of hypersensitivity to estradiol negative feedback and hypogonadotropism do occur in the young monkey, and they perhaps explain the long anovulatory menstrual cycles, which are characteristic of early adulthood and its periods of irregular adolescent sterility.[94]

It is important to point out that a prolonged steroid-independent (i.e., evident in ovariectomized monkeys) hiatus in GnRH secretion dominates the prepubertal period before menarche in the primate (see Chapter 32). Explanations for the regulatory mechanisms responsible for the prolonged hypogonadotropism in the premenarchial primate are of great interest, but they should neither overshadow nor be overshadowed by the gonadostat hypothesis because the latter mechanism relates only to the *final* stages of sexual maturation after menarche. During this period, steroid-independent drive to GnRH secretion is highly developed, but GnRH release remains suppressed until the response to the negative feedback actions of the low circulating concentrations of estradiol decreases, allowing for the final pubertal rise in GnRH to occur. Viewed from this perspective, both control systems would be used in the timing of first ovulations, with the steroid-independent system providing coarse control and the steroid-dependent system providing fine control. The steroid-independent control of GnRH secretion would determine the year when first ovulation is possible, while the steroid-dependent control mechanism would determine the week in which first ovulation occurs.

Similar to the altricial rhesus monkey, the precocial sheep has developed many components of the hypothalamo-hypophyseal-ovarian axis necessary for puberty (Stage III) within a few weeks after birth (Figure 31.9, bottom), such as a capacity for elevated secretion of GnRH/LH in the absence of the ovary. This ability to secrete GnRH/LH at a high rate, however, is quelled in the postnatal sheep through the estradiol hyperresponsiveness that dominates the vast majority of the developmental period until first ovulation at seven months of age. In contrast, for the monkey, the analogous period when sensitivity to steroid feedback inhibition is the primary regulator of LH secretion comprises only the final 25–30% of its postnatal development (~10 months between menarche at 30 months of age and first ovulation at 40 months) with the majority of the prepubertal hiatus in LH secretion due to steroid-independent inhibition (See Chapter 32). It is noteworthy that the developing sheep may have an analogous mechanism for nonsteroidal depression of GnRH secretion. However, because the sheep is a precocial species and is born in an advanced stage of reproductive development, this nonsteroidal inhibitory

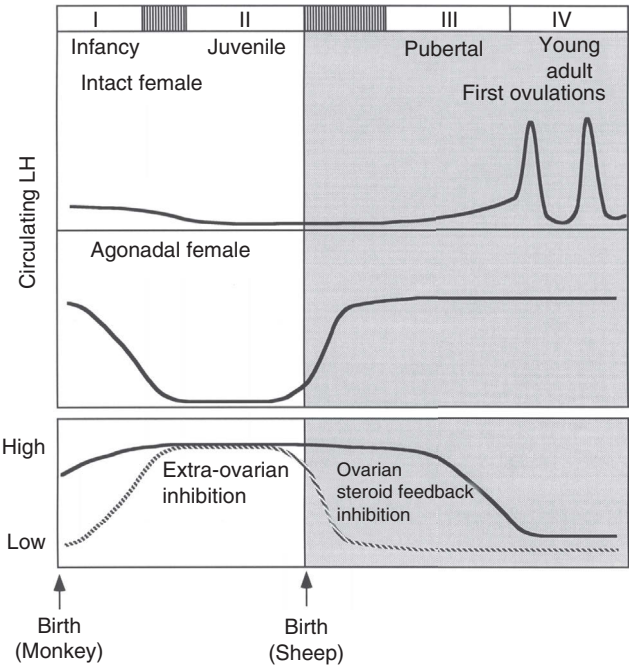

FIGURE 31.9 Analogous phases of sexual maturation based on the control of LH secretion in the monkey and sheep. The times of birth for each within the maturational scheme are indicated. Central to the hypothesis is that the sheep is born at a relatively advanced stage of sexual maturation, one that is comparable to that developed by the monkey shortly before menarche. *From Ref. 91, with permission.*

system is proposed to function before birth (see Refs 91 and 95 for reviews). Although this unifying hypothesis is attractive, it remains untested. Circulating LH in both sexes diminishes during the final stages of gestation in the absence of the gonad,[96] but the source of the "nongonadal" inhibition cannot be determined given that the fetus remains exposed to many exogenous substances, including maternal and placental steroids. The postnatal period of hypogonadotropism can be prolonged in the sheep if growth is retarded (see the section Energetics of Sexual Maturation). In such slowly growing lambs, removal of the gonads does not result in the high-frequency GnRH pulses typical of rapidly growing lambs. The issue of sensitivity to steroid feedback becomes important only after such animals have sufficient energy available to support the production of high-frequency GnRH secretion in the absence of any steroid feedback inhibition.

Events after the First LH Surge

An understanding of the first gonadotropin surge and the changes that precede this event is clearly important to unraveling the initiation of the pubertal process. Nevertheless, later changes occur in the sheep that are worthy of mention to provide the broad picture of the transition into adulthood. Sexual maturation continues well after the first ovulation; for example, the initial

ovulation is followed by a short luteal phase in some lambs because of premature release of $PGF_{2\alpha}$ from the uterus. There are also changes in the control of pulsatile LH secretion, initiation of normal sexual receptivity, and eventually a seasonal pattern of reproduction becomes manifest, with the young female returning to the anovulatory condition—much the same as before puberty.

Postpubertal Shift in Steroid Control of LH Secretion

During the pubertal period, the hypothalamo-hypophyseal system controlling pulsatile GnRH secretion decreases its ability to be inhibited by estradiol. High-frequency GnRH pulses do not occur indefinitely thereafter because a qualitative shift occurs in their steroid feedback control. The new inhibitory steroid is progesterone, and the new timing mechanism that dictates the intervals between ovulatory cycles is its source of secretion—the corpus luteum. According to the hypothesis developed for neuroendocrine control of the estrous cycle, rising luteal phase progesterone concentrations decrease GnRH pulse frequency (see Chapter 27). The resultant low-frequency LH pulses do not sufficiently stimulate the final stages of preovulatory follicle development. Following regression of the corpus luteum and the decline in circulating progesterone, LH pulse frequency again increases, and another 2- to 3-day follicular phase begins to set in motion the next reproductive cycle. This sequence of progesterone dominance and withdrawal is repeated at 15- to 17-day intervals (length of estrous cycle). The inverse relationship between circulating progesterone and LH pulse frequency during the first estrous cycles of the postpubertal female has been characterized.[27] As with the many other control systems, the potential for this feedback system to function exists well before puberty; experimentally, exogenous progesterone can decrease tonic LH secretion in the immature ovariectomized lamb.[45]

Sexual Receptivity

In the adult female sheep, progesterone plays an important "priming" role for estrogen induction of sexual behavior (see also Chapter 27).[97–100] As determined by treatment of the ovariectomized female sheep with physiologic patterns and levels of steroids, the sequence for induction of estrus is a sustained (at least 6 days) increase in circulating progesterone, withdrawal of progesterone, and an increase in circulating estradiol.[101] The requirement for this hormonal sequence may be used to explain why the first and even second ovulation of the lamb during the pubertal period is not accompanied by estrous behavior.[32,102] Before first ovulation, there is no corpus luteum, and hence there is no major increase in circulating progesterone to prime the neural centers that regulate sexual behavior to respond to the sustained estradiol rise that induces the first LH surge. The resultant short luteal phase generates only small amounts of progesterone, and therefore the increase in circulating estradiol before the second LH surge does not usually result in the expression of sexual behavior. Finally, after exposure of the brain to high concentrations of progesterone for several days during the first normal luteal phase, estrus first occurs in response to the rise in circulating estradiol typically during the third follicular phase. Therefore, as many as two "silent ovulations" (ovulations without estrus) may go unnoticed by the ram. Interestingly, estrus without ovulation has been reported in female lambs,[103] and this raises the question of whether the adult might have a more stringent requirement for progesterone priming. Other age-related differences have been noted in that the behavioral signs of sexual receptivity in the lamb, when they finally occur, are much weaker than in the fully mature female.[104] Moreover, the duration of estrus is shorter in lambs.[104–106] Nevertheless, a fertile mating can occur during the first reproductive cycles, and a pregnancy can be carried to term.[27,107] Compared to mature females, however, fertility of postpubertal lambs is lower (see Refs 22 and 23 for reviews). There is greater fertilization failure and embryo mortality,[104] and this has been attributed to a delay in development within the HPG axis, including the timing of ovulation relative to estrus and a lower viability of the ovum.[23]

Postpubertal Sterility—Seasonal Anestrus

Unequivocally, the greatest contributor to postpubertal infertility in the female sheep is seasonal anestrus.[108] Under natural conditions, the normal season of birth is spring. Puberty in spring-born lambs is attained between 25 and 35 weeks of age, after which estrous cycles occur at 15- to 17-day intervals. However, after only a few reproductive cycles, the young female becomes anovulatory and no longer exhibits estrous behavior. As determined through use of an experimental model, the ovariectomized estradiol-treated lamb, the female once again becomes hypersensitive to estradiol feedback inhibition of LH secretion. Thus, even before 1 year of age, the postpubertal lamb reverses its sensitivity to estradiol negative feedback and reverts to the anovulatory condition. This terminates the first breeding season.

The mechanism underlying seasonal cessation of estrous cycles in the sheep is hypogonadotropism due to insufficient GnRH secretion. The last cycle of the season occurs when the female attempts to drive the follicular phase, under increasing sensitivity to estradiol feedback inhibition that begins in late winter and early spring (also see the section Seasonal Reproduction in Adult Sheep). After luteal regression and the decline in progesterone, GnRH pulses start to increase and begin the next follicular phase, but the GnRH secretory mechanism

again becomes locked in a tight negative-feedback loop with estradiol. Under increasing estradiol hypersensitivity, the follicular phase cannot be completed because the high-frequency LH pulses required for the final developmental stages of the follicle necessary for ovulation cannot be produced. Consequently, not enough estradiol is secreted to activate the preovulatory gonadotropin surge system or induce estrous behavior. There is a marked seasonal change in the inhibitory feedback effect of estradiol on the pulsatile secretion of GnRH in adult ovariectomized female sheep.[109] During the breeding season, low doses of estradiol have no discernible inhibitory effect on GnRH secretion, whereas during the anestrous season, they produce a profound depression of GnRH pulse frequency.

DETERMINANTS TIMING THE TRANSITION INTO ADULTHOOD: THE CORE ISSUE

Clearly, the production of sustained high-frequency GnRH pulses initiates the sequence of events that activate the ovary. Consideration of the details of brain mechanisms involved in the production of these high GnRH pulse frequencies can be found in Chapters 11 and 27. The core question underlying the biology of puberty relates to what controls when this all-important increase in GnRH pulse frequency will occur. Is the timing by growth cues? How important are nongrowth cues? Is there a developmental clock regulated by genes? Why does puberty occur at different times in females and males? To begin to explore these fundamental issues, we can conveniently consider the sources of timing information as internal and external. Whatever explanation is ultimately forthcoming for the sheep, it must include how somatic signals communicate with the hypothalamus to control the developmental changes in sensitivity of GnRH secretion to inhibitory gonadal steroid feedback.

Internal Determinants

In virtually all living organisms, sufficient growth (and hence nutrition) is required to begin reproduction (Figure 31.10). However, we remain fundamentally ignorant of how growth is perceived by the central nervous system in any vertebrate species. The following sections are focused on potential humoral signals and neural mechanisms linking growth/nutrition with reproduction.

Energetics of Sexual Maturation

As noted earlier, Kennedy and Mitra[5] hypothesized 50 years ago that changes in energy availability serve as important cues timing puberty because the energy demands of thermoregulation and growth take

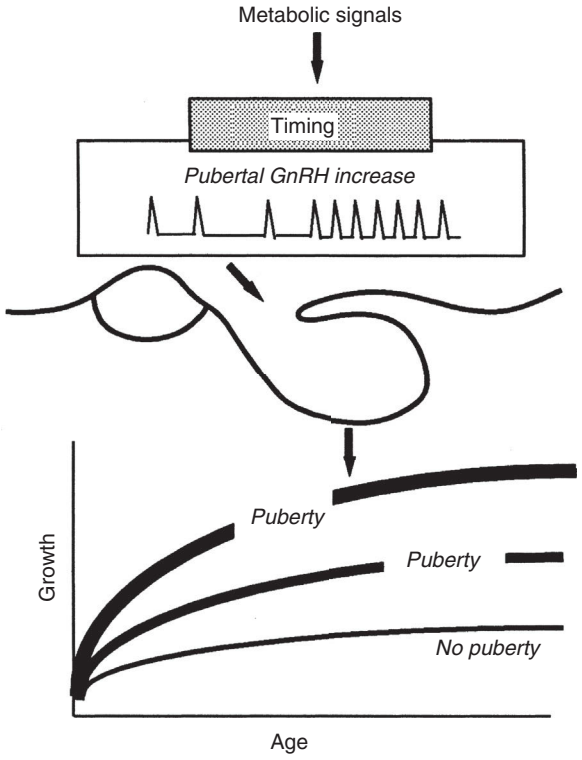

FIGURE 31.10 Growth and the timing of puberty.

precedence over that for reproductive function. As pointed out by Bronson and Manning,[110] the energetic cost of the physiological processes involved in ovulation are minuscule compared to the vastly higher energetic consequences of pregnancy and lactation. A similar argument could be made for the male, in which little energy is required for spermatogenesis compared to the great energy expenditure that occurs during intraspecies competition for females.

In view of the foregoing considerations, the energetics of the developing sheep can be considered. The newborn has almost no body fat[111,112] and therefore relies on milk from the mother for a continuous supply of energy. Compounding the lack of stored energy is its high energy expenditure. During early postnatal development, the ratio of large surface area to relatively small body mass results in rapid heat loss, and the metabolic rate must be correspondingly high to maintain core body temperature.[111,112] The greatest energetic cost is thermoregulation and cellular maintenance (Figure 31.11), and most of the remaining energy intake is allocated to muscular activity and growth. There is no excess energy as evidenced by the virtual absence of fat; consequently, according to the energetic hypothesis being developed, the priority for nonessential processes such as reproduction is low. The developing individual remains reproductively inactive due to hypogonadotropism. As body mass becomes larger with growth and the relative surface area

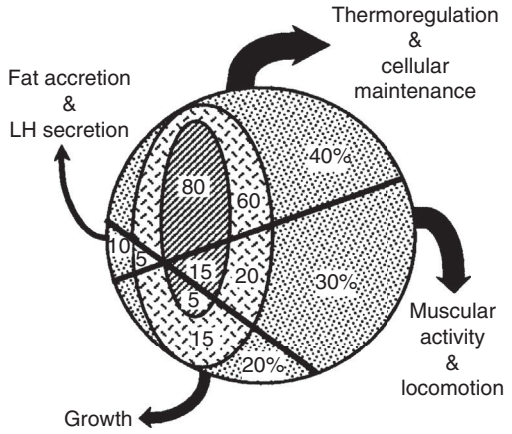

FIGURE 31.11 Partitioning of metabolic fuel during development in the sheep. The circles represent increasing body mass from birth (inner circle) to maturity (outer circle). Each circle is divided into four quadrants to represent the estimated relative proportion of energy allocated at each stage of development. *Modified from Bucholtz DC [Ph.D. Thesis]. University of Michigan.*

decreases, the metabolic rate correspondingly decreases. The eventual decline in metabolic rate allows more internal energy to become available for other physiologic demands, such as fat storage and, importantly, reproductive activities.

One potential approach to unravel how somatic signals influence brain pathways to govern GnRH secretion during sexual maturation is to study the influence of slow or restricted growth produced by restricted diet. With reduced energy intake, the majority of available energy is used for cell maintenance and thermoregulation, and very little remains for growth. Thus, there is a delay in reaching the transition point at which the metabolic rate declines and excess energy becomes available for fat deposition and reproduction—that is, puberty is delayed. Eventually, if growth is sufficient, some energy can be expended for the eventual consequences of reproduction (pregnancy/lactation or male–male competition). One could argue that studying the slowly growing or growth-retarded individual may not reveal mechanisms timing normal puberty; however, it is axiomatic that the rate of growth depends upon the available nutrition. Thus, because of large differences in food supply, a wide range of "normal" growth rates occurs in nature (outside the laboratory) for each species. Unfortunately, we have little definitive evidence on the sensors and pathways that link energy, metabolism, and reproduction via neural substrates. At the outset, we are presently hindered by an incomplete understanding of how the brain monitors energy metabolism both on a moment-to-moment basis and over the long term. Older work in rodents suggested that sensing of energy status may involve neurons in both the hypothalamus and brainstem[113,114] and that metabolic fuel sensing in the area postrema of the brainstem may play a role in energetic control of fertility.[115] However, the pathways that connect sensing of metabolic state to GnRH release are not well understood, and this is particularly true for the sheep. Candidate neuropeptides that may be involved will be discussed later in this section. Clearly, such knowledge is essential to provide an explanation of how the brain normally monitors growth to determine when it is sufficient to increase GnRH secretory activity to initiate puberty.

In the developing sheep, the influence of growth on GnRH secretion is readily apparent when the *ontogeny* of pulsatile LH secretion is examined in a population of lambs. Although the frequency of LH pulses increases in the neonatal period, the correlation is much greater with body weight (growth) than with age. This relationship is demonstrable in the presence[116] or absence (D.C. Bucholtz and D.L. Foster, unpublished observation) of the gonads. Perhaps such results reflect differences in energy supplies available to offspring before weaning, as the rates of lactation vary considerably between mothers. Experimentally, the relationship between LH pulse frequency and energy can be readily demonstrated by carefully controlling energy intake in lambs removed from their mothers and fed an artificial milk substitute.[117] Reducing the daily intake of calories to such suckling lambs profoundly retards the increase in LH pulse frequency. Eventually, they begin high-frequency LH secretion. However, with minimal fat stores (energy reserves), mechanisms controlling GnRH secretion remain energetically sensitive. Compared to rapidly growing peers that have accumulated sufficient energy reserves to withstand short-term (24-h) fasting, the slowly growing lambs are unable to do so. Short-term fasting of such lambs gradually depresses LH secretion. With realimentation, LH pulse frequency increases rapidly (i.e., within 4 h on the artificial milk diet). Remarkably, this increase can occur in some animals as quickly as within 20 min of refeeding. These findings suggest that without large energy reserves (fat) during development, mechanisms governing GnRH secretion are highly sensitive to changes in external sources of metabolic fuel (e.g., milk), even on a moment-to-moment basis. From a broader perspective, growth may influence the time course of sexual maturation via the accumulation of somatic fuel stores capable of sustaining GnRH secretion between meals.

Leptin as a Signal Linking Metabolism and Reproduction

What is the signal to the brain that relays energetic information to effect changes in reproductive activity? A long-held popular hypothesis, largely emanating from clinical literature and reviewed by Frisch,[118] is that a certain level of fatness must be achieved to begin reproductive cycles in the female. However, until the discovery of

the adipocyte-derived hormone leptin,[119] there had been no mechanistic hypothesis about how fatness per se could ultimately affect the activity of GnRH neurons. Leptin is a 16-kDa protein secreted primarily by adipocytes that acts within the hypothalamus to suppress appetite.[120,121] The first link between leptin and reproductive function was made after the observation that administering leptin to obese, hypogonadal, leptin-deficient *ob/ob* mice suppresses appetite, reduces body weight, and establishes fertility.[122] In adult sheep, peripheral concentrations of leptin are associated with measures of body fat,[123–125] and circulating leptin levels typically decrease during periods of reduced energy intake or availability[124–126] that are associated with reduced gonadotropin secretion. For leptin to serve as an important bloodborne metabolic signal timing the pubertal increase in GnRH secretion, two criteria must be satisfied: (1) puberty is associated with an altered pattern of peripheral leptin secretion; (2) exogenous leptin stimulates reproductive neuroendocrine function or advances the onset of puberty in otherwise sexually immature individuals.[127]

A developmental increase in peripheral concentrations of leptin has been reported for rodents and primates in some studies but not in others.[128–141] In addition, the pattern differs between males and females and may not always correspond temporally with indices of sexual maturation. In sheep, the pattern of leptin seems to depend on the sex, gonadal status, and nutritional conditions. In the neonatal lamb, serum concentrations of leptin are low (~2 ng/ml) as would be expected in view of its low amount of body fat.[142] An initial increase in circulating leptin occurs during the first week of life in both females and castrated males, and this is thought to result from the transfer of leptin from the mother to offspring during early lactation. Leptin then gradually declines between 1 and 7 weeks of age in both sexes. Throughout this pre-ruminant period (weaning typically occurs at 8–10 weeks of age), plasma leptin levels are regulated principally by level of nutrition.[143] In males reared from birth on an ad libitum milk substitute diet, plasma concentrations of leptin continuously increased as lambs grew to a body weight of 20 kg at approximately 8 weeks of age, and leptin was positively correlated with body fat as a percent of body weight. However, when lambs were fed milk substitute to provide a low plane of nutrition, plasma leptin did not increase and leptin was not correlated with the amount of body fat, even though the percentage of body weight attributable to fat increased with age. In a study of the patterns of LH secretion in males fed a solid diet of moderate energy, leptin concentrations increased between 6 and 12 weeks of age (L.M. Jackson, R.A. Ehrhardt, Y.R. Boisclair, and D.L. Foster, unpublished data). Leptin levels were positively correlated with body weight at 6 weeks of age (prepubertal), but the correlation was not significant at older ages. Plasma concentrations of leptin also correlated with the frequency of LH pulses in prepubertal males, which suggests that the growth-dependent increase in LH pulse frequency could be associated with an increase in plasma concentrations of leptin. Whether a causal relationship exists between peripheral leptin levels and the onset of high-frequency LH secretion in male sheep will require additional studies. Furthermore, peripheral leptin levels throughout development have not been characterized in female sheep; therefore, whether there are sex differences in the importance of leptin as a pubertal signal in this species is not known.

Regarding the second criterion for leptin as a pubertal signal, there is no consensus on the ability of exogenous leptin to stimulate gonadotropin secretion or advance puberty in sexually immature individuals. This could be due, in part, to the anorexigenic effects of leptin and the importance of adequate nutrition for activation of reproduction. In developing sheep, the effect of exogenous leptin on reproductive neuroendocrine function has been studied in both sexes. In prepubertal females, LH secretion was not stimulated after 8–10 days of recombinant ovine leptin treatment delivered either intracerebroventricularly (ICV)[144] or peripherally by intravenous infusion.[145] With peripheral leptin treatment, feed intake was not suppressed, but the ICV treatment dramatically suppressed feed intake to the extent that one would predict a decrease in LH secretion. One could speculate that because the expected reduction in LH pulses did not occur, the centrally delivered exogenous leptin actually preserved pulsatile LH secretion. Thus, there is no clear evidence that GnRH or LH secretion is stimulated by artificially increasing leptin levels in young female sheep.

In the developing male sheep, the ability of leptin to stimulate LH secretion appears to depend upon the stage of development and energy availability. The treatment paradigm used was previously shown to increase pulsatile LH secretion in a nutritionally hypogonadotropic adult model (gonadectomized, steroid-replaced, short-term fasted).[146] In this fasting model, subcutaneous administration of recombinant human leptin increases LH pulse frequency. Furthermore, leptin treatment is as effective at restoring reproductive neuroendocrine function as is refeeding. When the same dose of leptin that restored LH secretion in fasting adults was administered to prepubertal male lambs, LH pulse frequency increased, but the magnitude of the response depended upon individual pretreatment LH pulse frequencies.[146] These different patterns of gonadotropin secretion reflect the natural variability in levels of maturity at the same age. Leptin treatment did not increase the frequency of LH pulses in most lambs not yet producing LH pulses or in those individuals already producing hourly (or faster) pulses. However, in some lambs that were not producing

pulses before treatment and those lambs producing low-frequency pulses (1–3 pulses in 4 h), LH pulse frequency increased after leptin treatment. The ability of leptin to stimulate LH secretion in the prepubertal male contrasts with its effects in the female, which raises the possibility that there are sex differences in responses to growth cues timing the pubertal increase in GnRH secretion. However, as discussed below, female sheep are highly photoperiodic with respect to the timing of puberty, so it remains to be determined if the sex difference in the response to leptin in prepubertal individuals is due to differences in the responses to growth cues, photoperiod, or both.

The response in prepubertal male sheep to leptin differed from that in fasted adults. In all nutritionally hypogonadotropic adults, leptin increased LH secretion, but this was not the case in developmentally hypogonadotropic lambs. Such a finding suggests that there are additional developmental or metabolic signals that control the hypothalamo-pituitary response to leptin before puberty. Moreover, hypogonadotropism in the prepubertal individual experiencing limited energy availability due to rapid growth may not be functionally equivalent to nutritionally induced infertility in the adult. In the growth-retarded male lamb, responsiveness to leptin treatment does not improve with increasing age (Figure 31.12).[147] In fact, continued growth restriction reduced pulsatile LH secretion without leptin treatment and eliminated a neuroendocrine response to leptin in individuals who had been responsive at an earlier age when growth was normal or less severely restricted. These findings indicate that the mechanism regulating the onset of high-frequency GnRH secretion relies on one or more metabolic cues for information regarding growth and energy balance. Leptin can stimulate gonadotropin secretion in the prepubertal sheep, but the response appears to be regulated by other developmental or metabolic signals. Thus, this fat-derived hormone must be considered as a permissive signal timing puberty.

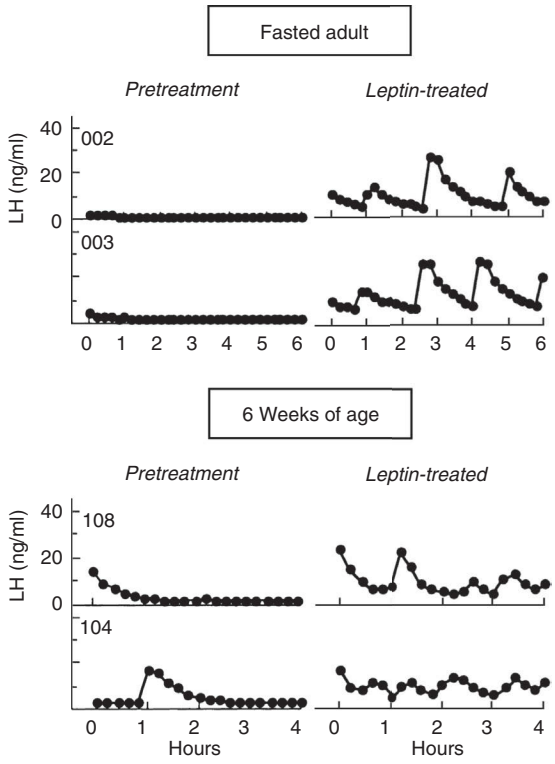

FIGURE 31.12 Patterns of LH secretion in representative gonadectomized, estradiol-treated male sheep before and after treatment with recombinant human leptin (12.5 μg/kg sc every 4 h). *Top*: LH pulse frequency is reduced in adults fasted for 48 h (*left*); 48 h of leptin treatment while continuing the fast increases LH secretion (*right*). *Bottom*: LH pulse frequency is low in nonfasted, growing lambs at 6 weeks of age (*left*); pulsatile LH secretion is increased after 72 h of leptin treatment (*right*) although responses were variable (see text for discussion). *Data from Ref. 146.*

Other Metabolic Signals

Before the discovery of leptin as a potential metabolic cue for puberty, the correlation of fatness with onset of fertility prompted other studies of the role of energy metabolites in timing puberty. Evidence for control of central mechanisms involved in the regulation of LH secretion by metabolic signals stems from studies using competitive inhibitors of glucose (2-deoxyglucose, 2DG) and free fatty acids (methyl-palmoxorate). As reviewed by Wade and Schneider,[148] such blockers were historically used for studies of appetite control, but their usefulness in the study of reproduction was later realized. For example, concurrent treatment with the inhibitors of both fatty acid and glucose oxidation in the hamster disrupts estrous cyclicity.[149] When this approach was applied to the developing sheep, pulsatile secretion of LH was suppressed within a few hours; interestingly, these treatments suppressed frequency in some lambs; in others, they suppressed amplitude.[150] Moreover, because the fatty acid blocker was administered after the glucose blocker, it is uncertain whether the major suppression of LH occurred in response to inhibition of glucose metabolism or fatty acid metabolism. In another study, when 2DG was administered alone, clear pulses of LH in the ovariectomized lamb were abolished.[151] Presumably, 2DG blocked the release of endogenous GnRH because both exogenous GnRH and a GnRH secretagogue (N-methyl-D, L-aspartate (NMDA)) were able to maintain pulsatile LH secretion when they were administered within 1 h of 2DG treatment. Such studies provide evidence for a role of energy balance in the moment-to-moment regulation of LH secretion. Interestingly, leptin may modulate glucose availability to provide information about energy balance because administration of 2DG blocks the ability of leptin to stimulate LH secretion during fasting.[152] Another possibility is that leptin, glucose, and perhaps

other substances serve as independent metabolic signals that are integrated within the brain to provide information about overall energy balance. If this is the case, perhaps a hierarchy of metabolic signals exists and the action of one signal is dependent upon the relative input provided by the remaining constellation of signals.

The metabolic hormone insulin-like growth factor-1 (IGF-1) has been proposed to be an important signal for puberty because circulating levels of the hormone increase during puberty in the rat,[153,154] monkey (see Refs 132 and 155 for reviews), and sheep.[156,157] In rodents, disruption of the growth hormone (GH)–IGF-1 axis by deletion of the gene for the GH receptor[158] or passive immunization against growth hormone-releasing hormone (GHRH)[159] reduces growth and delays sexual maturation. Furthermore, administration of IGF-1 increases peripheral concentrations of LH and advances puberty in the female rat[160]; in female monkeys, IGF-1 accelerates the decline in sensitivity to the negative feedback effects of estradiol[161] and advances the age at first ovulation.[162] In female lambs, the age of puberty is correlated with body weight and concentrations of circulating IGF-1 and the GH response to exogenous GHRH tends to be greater in those individuals reaching puberty at younger ages.[163] Similarly, plasma concentrations of IGF-1 in developing male lambs are positively correlated with testis size, plasma testosterone, and body weight, and IGF-1 levels are reduced in juvenile sheep during feed restriction and delayed puberty.[156] A direct effect of IGF-1 on the timing of puberty in sheep has not been studied, but chronic treatment with GH increases plasma concentrations of IGF-1 in prepubertal females and advances the age at which high-frequency (hourly) pulses of LH are produced.[164] In adult sheep, peripheral IGF-1 acutely increases LH secretion,[156] and the magnitude of the response is amplified in estradiol-treated or undernourished individuals. However, contrary to the rat,[165] the sheep hypothalamus appears to contain few IGF-1 receptors.[166] IGF-1 receptors and binding proteins are abundant in the ovine pituitary gland[167] and IGF-1 stimulates LH secretion in vitro from cultured ovine pituitary cells in a dose-dependent manner.[166] Furthermore, IGF-1 increases in peripheral circulation but not cerebrospinal fluid following an increase in nutrients that stimulates gonadotropin secretion,[168] and ICV administration of IGF-1 does not alter pulsatile LH secretion.[169] Thus, the major impact of IGF-1 on gonadotropin secretion in the sheep appears to be at the level of the pituitary gland.

Insulin is another metabolic hormone that has been proposed to regulate LH (GnRH) secretion (see Ref. 170 for a review). Specifically, low nutrition would result in low insulin concentrations that, in turn, would provide information to the GnRH neurosecretory system to reduce its function. This hypothesis has been tested in the growth-retarded lamb by acute injection of insulin into the lateral ventricles in an attempt to replicate improved energy balance and thereby to provide a positive signal for GnRH secretion.[171] Central administration of insulin minimized possible peripheral effects on glucose, amino acids, free fatty acids, and other metabolites that may affect neuroendocrine function. Acute injections of insulin failed to stimulate LH secretion which indicates that the increased circulating concentrations of insulin associated with increased feed intake after growth restriction may not be causal to the accompanying increase in LH secretion. Such findings do not support the role of peripheral insulin alone as an important signal mediating the effects of metabolism on GnRH secretion in the lamb. However, in another study, mean LH concentrations increased after two and four days of ICV infusion of insulin to diet-restricted, pubertal female sheep,[172] despite the hypophagia induced by the insulin treatment. The difference between the responses to acute and chronic insulin treatment raises the possibility that central levels of insulin must be integrated over time or with other metabolic factors to provide information about nutrient status to the GnRH neuronal system.

Pathways of Energy Metabolism in Modulating GnRH Secretion

While the energetic state of an individual clearly plays a role in timing puberty onset, the neural pathways whereby nutritional status is transduced into a signal influencing LH secretion are not completely known. One commonly used approach to uncover neural pathways involved in mediating the effects of nutrition on GnRH/LH secretion is acute or chronic feed restriction. Various lines of evidence indicate that the reduced gonadotropin secretion in the growth-retarded lamb is specifically due to reduced GnRH secretion (Figure 31.13). First, the frequency of GnRH pulses, as measured in the pituitary portal vasculature, is markedly reduced in growth-retarded lambs compared to rapidly growing lambs.[174] Second, GnRH content in the median eminence (terminals) and preoptic area (cell bodies) is not diminished in growth-retarded lambs.[175] Third, hourly, intravenous administration of the glutamate analog, NMDA, to nutritionally growth-retarded lambs produces corresponding LH pulses that are similar in size to those induced by a physiologic dose of GnRH.[175] Fourth, the anterior pituitary gland contains adequate concentrations of LH and FSH,[176] and physiologic doses of GnRH readily induce LH and FSH secretion.[175] These findings, considered together, lead to the inference that the central mechanisms controlling GnRH release, rather than synthesis of GnRH or LH, are limiting LH secretion when sexual maturation is delayed by growth retardation. The following paragraphs will discuss a few of the more likely central neuropeptide candidates that may be involved and for which there is data available in the sheep.

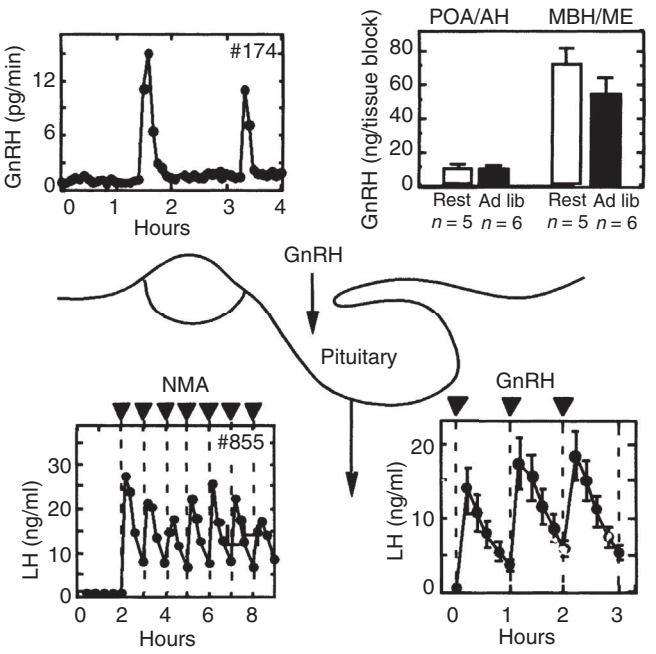

FIGURE 31.13 Central mechanisms controlling pulsatile LH secretion in the nutritionally growth-restricted female lamb. See text for explanation of insets. *Top*: Pattern of GnRH in pituitary portal blood of a representative growth-retarded lamb (*left*). Mean (±SEM) hypothalamic GnRH content in lambs fed restricted (Rest) and ad libitum (Ad lib) diets; POA/AH, preoptic area/anterior hypothalamus; MBH/M, mediobasal hypothalamus/median eminence (*right*). *Bottom*: Patterns of LH secretion in a representative growth-restricted lamb injected with NMDA (5 mg/kg BW) (*left*). Mean (±SEM) LH concentrations in lambs injected with GnRH (5 ng/kg BW, $n = 15$) (*right*). *Redrawn from Ref. 173; portal GnRH data from Ref. 174.*

There are several neurotransmitter/neuropeptide systems that regulate GnRH secretion and that are also altered during nutrition-induced growth retardation and its resultant hypogonadotropism. Increased inhibition by endogenous opioids was one of the first mechanisms postulated whereby metabolic changes influence the pulsatile secretion of GnRH. In the rat, there is limited evidence that fasting impairs LH secretion by activating an endogenous opioid pathway.[177,178] In the growth-restricted lamb, however, such evidence has not been obtained. Although endogenous opioids appear to be involved tonically in the steroid-independent inhibition of LH secretion in the lamb as discussed previously (in the section Decrease in sensitivity to estradiol feedback inhibition of GnRH secretion during puberty), whether this inhibition is heightened during growth retardation is uncertain. Blockade of endogenous opioid receptors with naloxone in the hypogonadotropic gonadectomized lamb does not increase LH pulse frequency at doses that are effective in this regard in the rapidly growing agonadal female. Even greater doses of naloxone[175] or the long-acting antagonist, naltrexone (H. I. Anson and D.L. Foster, unpublished observation), have not been able to disinhibit LH secretion. One explanation is that other inhibitory neuronal pathways are also activated during periods of reduced energy metabolism. Thus, opioidergic inhibition of GnRH secretion may occur, but it would be masked by one or more other inhibitory neurotransmitter pathways, and blockade of only opioid receptors by an antagonist would be without an effect on GnRH secretion. The use of multiple pharmacologic agents, along with the opioid antagonist, would be required to test this possibility. Nevertheless, based on the existing evidence, we conclude that a massive increase in opioid tone cannot be the primary cause of hypogonadotropism in the growth-retarded lamb.

Neuropeptide-Y (NPY) is another candidate neurotransmitter for modulation of GnRH secretion during low nutrition. Hypothalamic NPY neurons are responsive to metabolic signals as evidenced by co-localization of leptin receptors on NPY neurons in rodents[179] and nonhuman primates,[180] and a reduction in hypothalamic NPY mRNA expression[181,182] and NPY secretion[182] in response to leptin treatment. Expression of leptin receptor mRNA has been reported in the arcuate nucleus in the sheep, an area that also contains many NPY neurons.[183] Concentrations of NPY in the cerebrospinal fluid are higher in food-restricted, ovariectomized, estradiol-replaced lambs than in well-nourished lambs[184] and, in ovariectomized sheep, ICV infusion of NPY decreases LH secretion.[184–186] Because ICV injections of NPY also stimulate feeding in sheep,[187,188] as in other species (see Ref. 189 for a review), the neuropeptide may serve a dual role in the pathway of signal transduction that conveys information about metabolic cues to the neuronal systems controlling GnRH secretion and ingestive behavior. In a prepubertal individual, elevated NPY levels would suppress GnRH secretion until circulating metabolic signals (e.g., leptin) reflect somatic maturation, at which point the inhibitory NPY input would diminish and GnRH secretion would increase. NPY neurons also coexpress agouti-related peptide (AgRP) and AgRP also increases with feed restriction.[190–192] Reports regarding the effect of AgRP on reproduction generally report an inhibitory function,[193–195] although a stimulatory effect in male mice was noted in an early study.[196] Data regarding the role of AgRP in regulating LH secretion in the sheep is sparse, but an inhibitory function fits with one report wherein AgRP mRNA expression was negatively correlated with LH release in sheep on a decreasing plane of nutrition.[197] As AgRP is an endogenous antagonist to α-melanocyte stimulating hormone (αMSH) and αMSH appears to be stimulatory to LH release (see below), AgRP may act to inhibit αMSH input and thereby reduce GnRH/LH secretion.

αMSH is produced as one of several products of the proopiomelanocortin (POMC) gene. Proopiomelanocortin gene expression decreases with food restriction and is a direct target of leptin, which stimulates its expression.

αMSH acts through various isoforms of the melanocortin receptor (MCR), of which MCR-4 is considered the most important for body weight regulation.[198] As mentioned above, both αMSH and AgRP bind to MCR-4 in antagonistic fashion, and the fact that deletion of these receptors leads to obesity and hyperphagia[199] strongly suggests that αMSH predominates in that relationship. Activation of MCR-4 is generally considered stimulatory to reproduction[200–203]; in sheep, administration of MTII, an agonist of MCR-4, increases LH in the luteal phase.[204] Whether these effects are directly exerted at the GnRH neuron,[195,205] exerted indirectly,[204] or both remains to be determined.

Kisspeptin-containing neurons of the arcuate nucleus may also play a role. Kisspeptin expression in rodents is reduced by food restriction[206–210] and is reduced in male mice with a deficiency of leptin.[211] Arcuate kisspeptin neurons, but not those in the preoptic area, coexpress the receptor for leptin (although to what extent is a matter of contention[211–214]), as well as receptors for insulin.[215] Given these findings and the importance of the kisspeptin system to puberty onset, it is not suprising that they are a topic of great interest where metabolic regulation of reproduction is concerned. In mice, deletion of leptin receptors from kisspeptin neurons had no affect on puberty onset[216] and reintroducing leptin receptors to kisspeptin neurons in mice globally lacking leptin receptors (*db/db* mice) did not restore fertility.[217] Interestingly, deleting insulin receptors from kisspeptin neurons delayed onset of puberty.[215] In sheep, *KISS1* mRNA expression is lower in female sheep fed to be lean as compared to females of a normal body weight and is partially restored by leptin treatment.[218] These authors also reported that all kisspeptin neurons examined in that study coexpressed leptin receptor. They further reported reciprocal connections between kisspeptin neurons and both NPY and POMC neurons and that kisspeptin treatment increased NPY mRNA, but reduced POMC mRNA levels. Given the respective effects of NPY and αMSH on LH secretion, this would seem counterintuitive. Thus, the exact nature of such interactions in the sheep awaits further clarification.

External Determinants Timing Puberty

Because the sheep is a seasonally breeding species, it is logical to consider that external cues would be integrated with internal, growth-related cues to modulate the activity of the GnRH pulse generator (Figure 31.14). These cues might provide conflicting information. For example, if environmental information indicates that the season for puberty is not optimal, the expression of high-frequency GnRH pulses will not be permitted, despite the fact that growth-related information indicates that an adequate size or sufficient energy balance for puberty has been attained. Similarly, sexual maturity will not occur if seasonal cues indicate that the appropriate time of the year for puberty has arrived, but metabolic cues indicate that the lamb cannot bear the energetic consequences of increasing GnRH pulse frequency and initiating reproductive function. The sustained increase in GnRH pulse frequency will occur only when internal and external cues indicate that both size (positive energy balance) and season are favorable for initiating a pregnancy. To appreciate mechanisms of how seasonal cues impact neuroendocrine mechanisms regulating the onset of fertility in the female lamb, the seasonal nature of reproduction in the adult female sheep is considered below (also see Chapter 34; Refs 219 and 220 for reviews).

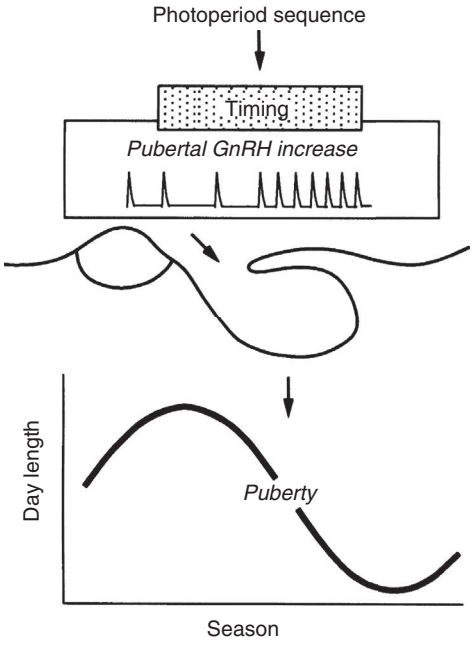

FIGURE 31.14 Photoperiod and the timing of puberty.

Seasonal Reproduction in Adult Sheep

Although the sheep has long been associated with humans, it retains a seasonal pattern of reproduction. Conception is restricted to a certain period of the year—the *breeding season*. During the remaining *anestrous season*, the female is anovulatory and will not exhibit estrous behavior. As with other seasonal breeders, the ability of the sheep to turn the reproductive system off and on, a process of reversible fertility, may be viewed as "nature's own contraceptive."[221] Because the length of pregnancy in female sheep is about 5 months, the breeding season occurs in autumn and winter so that the births of lambs are clustered in spring, the "season of plenty." This reproductive strategy allows access to high-quality forage, both to meet the demands of lactation for the adult and to meet the subsequent growth requirements of the postweaning lamb. As will be pointed out later, birth

in spring is advantageous in another regard. It allows the majority of postnatal development to occur during spring and summer so that puberty can occur during the same year, in the first autumn after birth. Artificial selection and better nutrition have extended the breeding season, and now a gradation exists from the monoestrous state of some wild species to the polyestrous condition of domestic breeds.[106] Various breeds are capable of mating for as many as 8–10 months of the year,[222,223] and some individuals have even been reported to exhibit estrous cycles at 15- to 17-day intervals throughout the year.[107,224] This suggests that the potential for continuous reproduction exists in the sheep, and artificial selection could make this possible. Presently, assisted reproductive technologies, including hormonal manipulations, facilitate conception and birth of lambs at any time of the year if so desired.

Numerous types of experiments have demonstrated that day length is the primary environmental factor timing the annual reproductive cycle of the sheep. Originally, Marshall[225] determined that transferring female sheep across the equator to a locale where environmental variables were 6 months out of phase eventually shifted the breeding season 6 months. More definitively, reversal of the annual changes in only day length by artificial photoperiod from that which occurs under natural conditions reverses the breeding and anestrous seasons.[226–228] Acceleration of the changes in the annual photoperiod, by alternating 90-day cycles of long days and short days, also accelerates the annual reproductive cycle to produce two breeding seasons and two anestrous seasons per year.[229] Finally, manipulation of the circadian pattern of melatonin, the pineal hormone involved in transducing photoperiodic information into a neuroendocrine signal, results in changes in timing of when reproductive cycles occur (see Ref. 220 for a review). These types of investigations in which photoperiod or its pathway of transmission into neuroendocrine signals is changed offer compelling evidence for a deterministic role for day length in synchronizing breeding activity among mature female sheep. As discussed below, this pathway is also used by the developing female to synchronize its first reproductive cycles to the autumnal breeding season of the adult.

Photoperiod and Puberty

The month of birth will influence the age of puberty, and this is most evident in those breeds that exhibit the most pronounced seasonal reproduction. The classic study by Hammond[2] in 1944 led to the general conclusion that regardless of season of birth, lambs would reach puberty only during the breeding season. Moreover, it has been noted in several breeds of sheep that winter-born and spring-born lambs reach puberty at about the same time of the year; however, because the latter are born several months later, they are much younger at the first mating.[2,106,107,223,230] Births later in the year (summer) may even further reduce the age at puberty.[31,223,231] However, if births occur extremely late in the year (autumn), lambs delay initiation of reproductive cycles until the following year to coincide with the subsequent breeding season.[2,107,223,230,232]

Long-standing evidence that photoperiod is the major environmental factor phasing the annual rhythm of reproduction in the adult female led to the consideration that seasonal changes in day length might also serve as the outdoor cue to synchronize the time of puberty to the appropriate season for first conception. This is summarized in Figure 31.15. According to this scheme, the

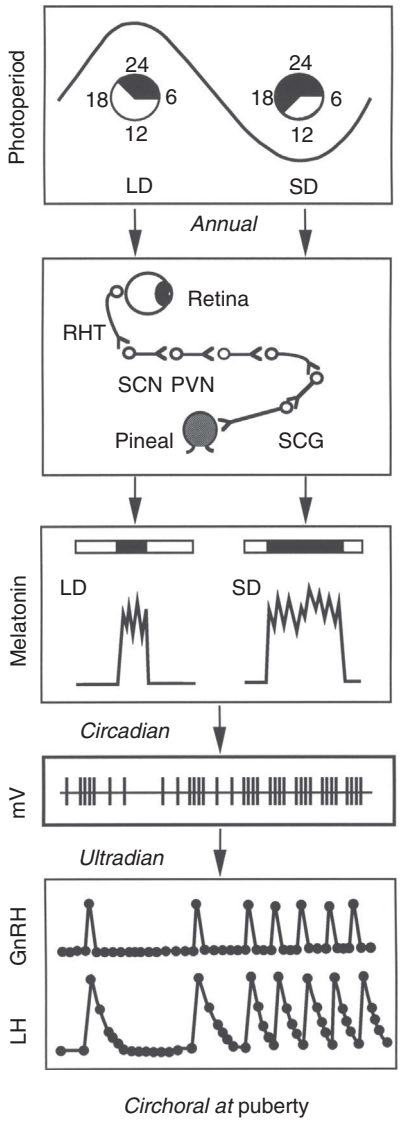

FIGURE 31.15 Interplay among rhythms timing puberty involving day length, the pineal gland, electrical activity of GnRH neurons, and GnRH/LH secretion. LD, long day; SD, short day; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; SCG, superior cervical ganglion. *Redrawn from Ref. 233.*

developing sheep maintains a photoperiod history from a very early age using the same photoneuroendocrine pathway as the adult to transduce seasonal changes in day length into humoral signals that eventually modulate neuroendocrine function. The long days of spring and summer, followed by the decreasing day length of autumn, provide the stimulatory (permissive) photoperiod sequence to initiate puberty. As reviewed elsewhere for the adult,[219] information about length of day (hours of light vs hours of darkness) is relayed from the retina through a retinohypothalamic tract via the suprachiasmatic nuclei, which provide circadian input; the information proceeds through the paraventricular nuclei, and then via a multi-synaptic pathway involving the superior cervical ganglia to the pineal gland. Thus, the environmental light/dark cycle entrains the circadian-based melatonin rhythm such that melatonin is only secreted for the duration of the dark phase of the photoperiod. Long days produce a short duration of high melatonin secretion, whereas short days induce a long duration. These patterns of melatonin form the basis of a neuroendocrine record of photoperiod experience that modulates the frequency of GnRH secretion.

Evidence for key elements of the foregoing integrated mechanism that will be discussed in detail next includes: (1) a seasonally reversed artificial photoperiod sequence can reverse seasonally delayed puberty; (2) normal puberty requires the experience of long days before short days; (3) photoperiodically delayed puberty is associated with slow LH pulse frequency due to prolonged hypersensitivity to estradiol negative feedback; and (4) removal of pineal gland function delays puberty, and timed infusions of its product, melatonin, restore the time of puberty to normal.

SPRING-BORN VS AUTUMN-BORN LAMB—A CASE OF DELAYED PUBERTY

A study of the age of puberty in lambs (Suffolk breed) born in spring or in autumn was one of the first to implicate photoperiod as an important environmental timer of puberty.[232] In that study shown in Figure 31.16, onset of cycles was determined by estrous behavior using mature vasectomized males housed continuously with the lambs; the increase in circulating progesterone to luteal phase concentrations confirmed that reproductive cycles had begun. In natural photoperiod, spring-born lambs attained puberty at about 30 weeks of age during the breeding season (Figure 31.16, second panel). By contrast, autumn-born lambs raised in natural photoperiod reach the pubertal age of 30 weeks during the anestrous season; reproductive cycles did not occur in such females (Figure 31.16, third panel) despite their having sufficient somatic development for initiation of ovulations. Rather, puberty was delayed until the following breeding season, when the lambs were nearly 1 year old. This seasonally induced delay in onset of reproductive cycles can be attributed to day length. Evidence supporting this inference is that the age at puberty was restored to normal in autumn-born females when they were raised in an artificial, annually reversed photoperiod simulating that had they been born in spring (Figure 31.16, bottom panels).

Although the foregoing study provides evidence that lambs of the Suffolk breed are highly photoperiodic with respect to the timing of puberty, this may not be the case in all breeds. This is shown in Figure 31.17 in a study of the effect of season on timing of puberty in the D'man breed.[234] The D'man is a subtropical sheep that has evolved under a highly intensified production system in the oases of pre-Saharan regions, and it has a pattern of relatively continuous reproduction (90% conception rate in both spring and autumn).[1,235] Thus, it provides

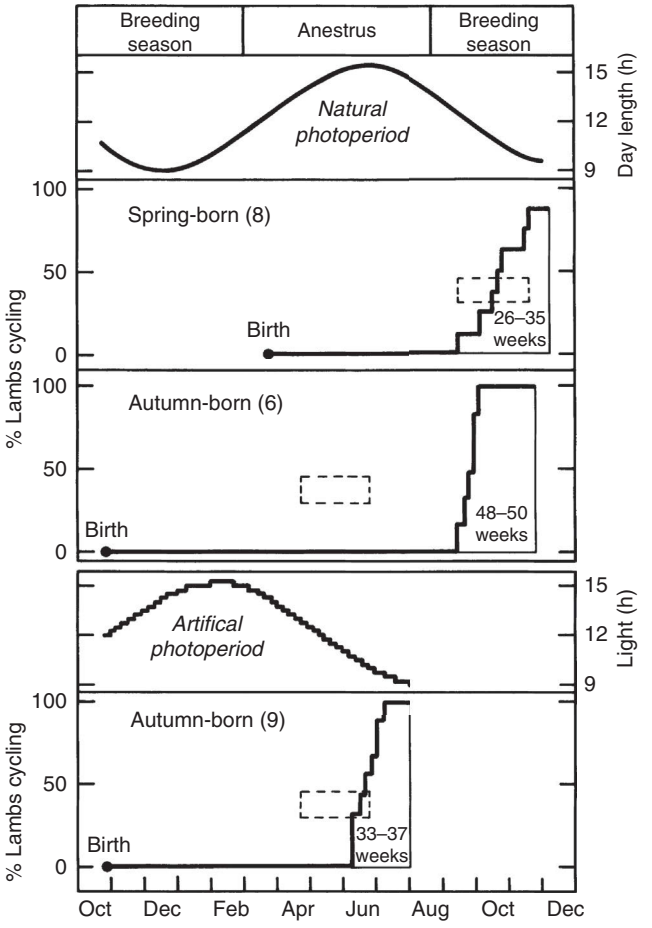

FIGURE 31.16 Delay in puberty in autumn-born lambs due to inappropriate photoperiod during development. Shown are the ages and months at first ovulation in spring-born and autumn-born females raised in natural photoperiod (*first three panels*) and in autumn-born females reared in an artificial, seasonally reversed photoperiod (*last two panels*). Ovulations were initiated in spring-born females during the age range of 26–35 weeks (*dashed rectangle*). *From Ref. 20, with permission.*

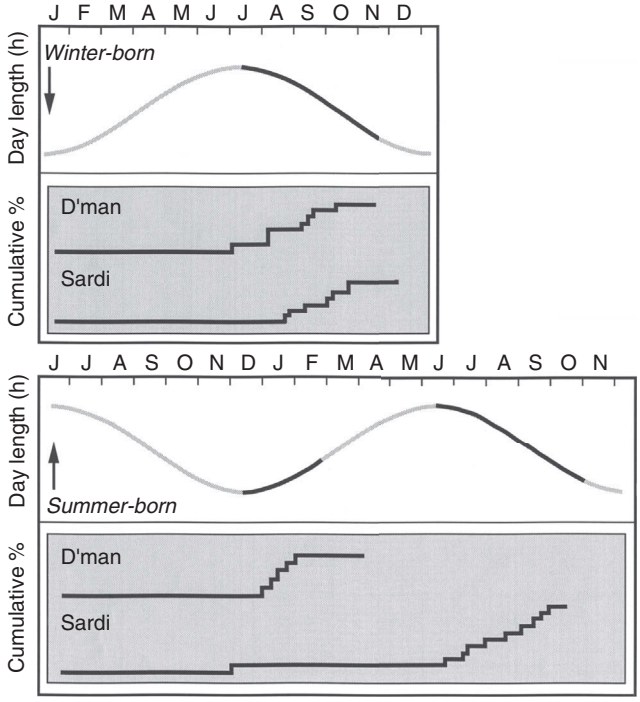

FIGURE 31.17 Timing of puberty, as evidenced by the first rise in circulating progesterone to luteal phase concentrations in lambs of the D'man breed and of the Sardi breed. Lambs were born either in winter (*top*) or summer (*bottom*) and raised in natural environmental conditions in Morocco. *Data from Ref. 234, with permission.*

an exception to the rule that reproduction in the sheep is highly seasonal. As a comparison to the D'man breed in the same area (Morocco), the Sardi breed is raised under much more open conditions and is seasonal in its pattern of reproduction. In this Moroccan study[234] conducted under natural photoperiods, conception was regulated so that lambs of both breeds were born in two different periods of the year, January and June. As expected, winter-born lambs of both breeds attained puberty as evidenced by initiation of luteal function in the autumn, much the same as the Suffolk lamb as shown in Figure 31.16. However, in the strongly seasonal Sardi breed, lambs born in the summer delayed puberty, again much like the autumn-born Suffolk female. By contrast, summer-born lambs of the D'man breed initiated reproductive cycles at the same age as those born in the winter. Taken together with information about seasonality of reproduction in the adults of these breeds, one is led to the conclusion that seasonal cues only play a role in timing of puberty if reproduction in the adult is seasonal.

The photoperiod is used to time puberty through the timing of the decrease in sensitivity to estradiol inhibition of LH secretion. This is shown in Figure 31.18 for the ovariectomized model in which the potential to produce high-frequency LH pulses typical of the follicular phase is revealed at an early age (e.g., by 12 weeks of age). In the presence of physiologic levels of circulating estradiol (by sc implant), only a low frequency is maintained; the time when high-frequency pulses are expressed depends upon photoperiod history. The lamb raised in a stimulatory (i.e., permissive) photoperiod does not express the high-frequency LH pulses until 25–30 weeks of age in the presence of estradiol (top panels, ovariectomized (OVX) + estradiol (E)), despite its having achieved this potential at a much earlier age (top panels, OVX). However, the lamb raised in an inhibitory photoperiod does not reduce its sensitivity to estrogen feedback inhibition at the same time as the lamb raised under the stimulatory photoperiod, and LH pulse frequency remains suppressed for much longer (bottom panels, OVX+E).

In the context of season of birth and the timing of puberty (Figure 31.16), the delay in initiation of reproductive cycles in the autumn-born lamb reared outdoors arises from prolonged hypersensitivity to estradiol negative feedback.[232] Whereas the response to estradiol feedback inhibition of LH secretion diminishes in the spring-born lamb at 30 weeks of age during decreasing day length, the autumn-born female remains hypersensitive to estradiol at this age because of increasing day length. This maintains tonic LH secretion at low levels, thereby preventing the initiation of preovulatory follicular development (and subsequent pregnancy) during the long days of summer in such females born in the "wrong" season. The reduction in feedback sensitivity then occurs during the decreasing day length of autumn to synchronize puberty to the breeding season. Finally, this delay in reduction of sensitivity to estradiol negative feedback can be prevented experimentally. When the autumn-born female is raised indoors in an artificial, annually reversed photocycle, the decrease in feedback sensitivity is restored to the normal age (~30 weeks).[232] Therefore, photoperiod cues, as well as growth-related cues, are used to time the initiation of ovulations in the developing sheep.

Studies of neuroendocrine timing of puberty in nonseasonal breeds of sheep have only begun. In this regard, the pattern of LH secretion during puberty in Sardi (strong seasonal breeder) and in D'man (aseasonal) accords with the timing of puberty shown in Figure 31.17. In the D'man, the increase in LH secretion was the same regardless of whether the lambs were born in winter or summer. By contrast, in the summer-born Sardi female, much like the Suffolk female, the increase in LH pulse frequency did not occur at the usual age, and was delayed until the next breeding season. Thus, in some breeds of sheep, only growth cues may be used to time puberty, whereas in others, both growth cues and photoperiod cues are important (L. Derqaoui, unpublished observation[234]). Studies of female Soay sheep using photoperiodic and growth manipulations suggest that, within the body weight range examined, photoperiod is the dominant cue timing puberty in this less

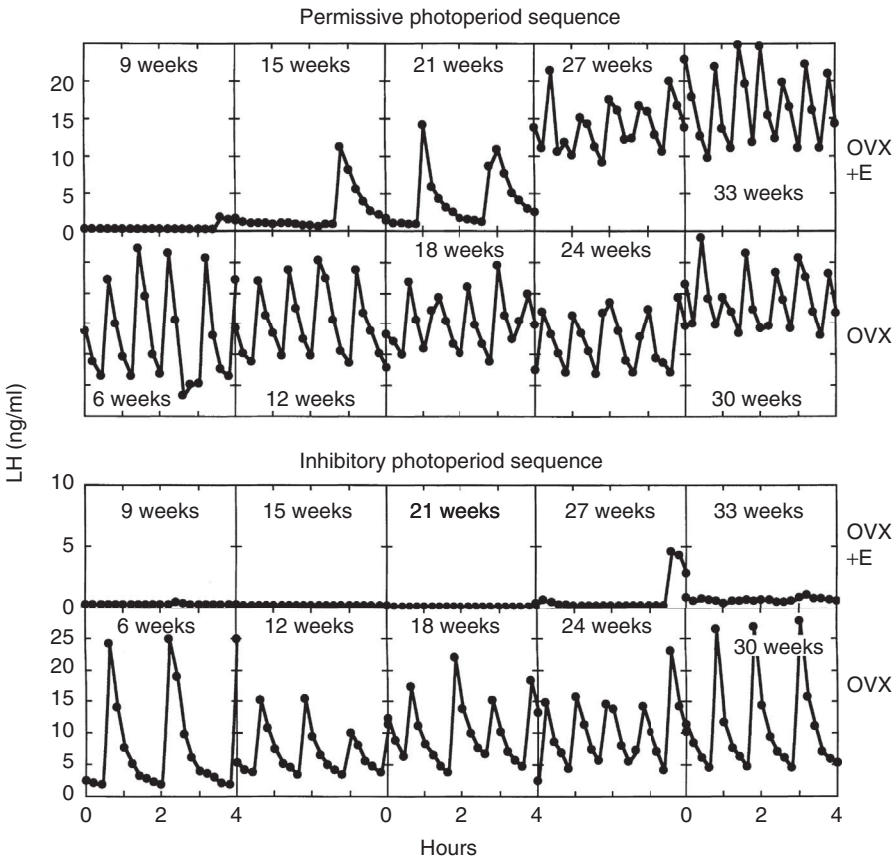

FIGURE 31.18 LH patterns at different ages in a lamb maintained in a permissive photoperiod sequence for puberty (*top two panels*) and in a lamb maintained in an inhibitory photoperiod sequence for puberty (*bottom two panels*). For each lamb, upper panels depict LH patterns 3 weeks after replacement of a constant release estradiol capsule; lower panels depict LH patterns 3 weeks after removal of estradiol. *From Ref. 44, with permission.*

domesticated breed.[236] This finding that photoperiod can induce puberty in Soay females with very limited growth while the same degree of growth restriction delays puberty in Suffolk females, could simply reflect an extremely low threshold for the energy balance required to initiate high-frequency GnRH secretion in the more feral breed. However, it could also represent an adaptation to harsh environmental conditions in which adequate forage during lactation and weaning are critical for offspring survival. Strict adherence to photoperiodic cues for timing puberty would optimize reproductive success by minimizing the number of births that would occur during late winter or early spring when food availability is unpredictable.

PHOTOPERIOD SEQUENCE FOR PUBERTY

The simplest hypothesis for photoperiodic timing of puberty in the female sheep is that the lamb can begin reproductive cycles during short days, but not during long days. Thus, the spring-born lamb could monitor photoperiod, and when day length becomes sufficiently short in the autumn, puberty occurs. Indeed, females raised from birth under long daily artificial photoperiods do not begin reproductive cycles during the normal age (25–35 weeks).[237] Eventually, ovulations began during the second year, although in many long-day-reared lambs they occur at irregular intervals, and many result in short luteal phases. Although the latter finding importantly indicates that long days can block the normal timing of puberty, its eventual onset implies that the lamb is able to reject photoperiod information (become photorefractory) so that puberty cannot be forestalled indefinitely in an unfavorable light environment. Apparently, after growth requirements have been satisfied, the window of opportunity to achieve fertility can be shifted, but not closed entirely. This underscores the timing role, rather than driving role, played by photoperiod in puberty in the female sheep.

An unexpected finding in a series of photoperiod studies was that exposure of the female sheep to short days alone delays puberty, much the same as long days. When lambs are raised from birth under continuous short days, repetitive ovulations do not even begin during the first year after birth (Figure 31.19, top). It appears that lambs must experience alternate photoperiods to begin reproductive cycles at the appropriate age. Specifically, long days must precede the short days during which puberty is attained. Systematically reducing the duration of long-day exposure in the study shown in Figure 31.19 revealed that as little as 1 week would induce reproductive cycles in lambs otherwise raised in short days. Clearly, the photoperiodic timing of puberty in the female lamb is much more complex than the simple notion that the mere experience of short days alone

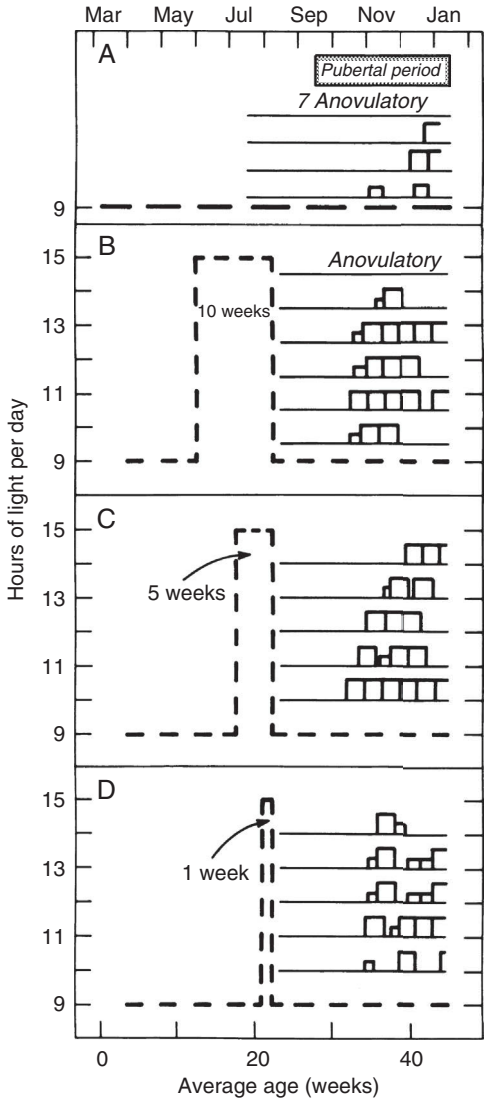

FIGURE 31.19 Alternate photoperiods initiate reproductive cycles. Spring-born lambs were raised entirely in artificial short days of 9 h light: 15 h dark (9L:15D) or with 10 weeks, 5 weeks, or 1 week of long days of 15 h light: 9 h dark (15L:9D). Reproductive cycles with normal luteal phases (*large blocks*); cycles with short luteal phases (*small blocks*); photoperiod (*broken lines*). Each horizontal line shows data for an individual lamb, except in the top panel, which indicates that seven of 10 lambs remained anovulatory. *From Ref. 42, with permission.*

initiates reproductive cycles. The finding of a long-day requirement to begin ovulations in this young short-day breeder raises the possibility that the lamb maintains a *photoperiod history* and that it uses the long days of spring and summer as a contrast reference to time puberty to the relatively shorter days of autumn.

PINEAL GLAND AND MELATONIN

In the adult sheep, melatonin plays an important role in the timing of seasonal reproduction. It is likely that it has a similar role in the lamb to regulate the photoperiodic timing of puberty. Important sites for melatonin action appear to be in the hypothalamus, but uncertainty exists about the mechanisms that translate information about the pattern of melatonin to alter the pattern of GnRH secretion (see Chapter 34). Using microimplants of melatonin into various areas of the ovine brain, early studies identified targets for melatonin action that affected LH secretion in the mediobasal hypothalamus[238] and premammillary region.[239] Interestingly, several studies[240–243] have reported binding sites of melatonin in the pars tuberalis of the pituitary, and more recent work indicates that melatonin action in this region may well be critical for seasonal breeding (see Chapter 34 for detailed discussions of this mechanism). The issue of a timekeeping relay is essential to our understanding of melatonin action because of the long lag time (several weeks) between changes in the pattern of melatonin secretion and the appropriate LH response.[219,233] Moreover, studies in adult female sheep have found that melatonin does not have an acute action (within minutes) in the modulation of LH secretion.[244] In view of the foregoing considerations about the long time constant, it is possible that melatonin modulates GnRH secretion through inducing changes in morphology by remodeling yet unknown elements within the brain. The lack of exact understanding about how melatonin interacts with mechanisms controlling GnRH secretion, however, should not detract from the important concept that the pattern of circulating melatonin encodes day length. This information is then used to increase or decrease the sensitivity of the GnRH neurosecretory system to estradiol negative feedback to alter the frequency of LH secretion.

The sheep is exposed to the photoperiod-linked melatonin rhythm very early in life. Melatonin has been detected in the circulation of the fetus, and the pattern exhibits a well-defined rhythm, with the highest levels occurring during the hours of darkness.[245,246] The pattern in the fetus mimics that of the mother, although its amplitude is reduced. This is for good reason because the mother generates the rhythm as evidenced by the observation that removal of the maternal pineal abolishes the nocturnal increases in melatonin in both the maternal and fetal circulation.[247,248] This leads to the conclusion that the fetal pineal gland may not produce melatonin and that the melatonin in the fetal circulation is derived from the maternal pineal gland. Such a conclusion is reinforced by the finding that exogenous melatonin can cross the placenta from the maternal to the fetal side.[247–249] However, melatonin has been detected in the fetal pineal gland[245] raising the possibility that melatonin could be secreted in very small quantities before birth because the pineal gland does not store melatonin appreciably.[250] In view of the depressed melatonin in fetuses of pinealectomized mothers, however, the secretion of

melatonin by the fetal pineal may be insignificant. Moreover, if the fetal pineal were to secrete melatonin, then the circadian pattern of secretion would not accurately reflect day length because it is not obvious how the fetus would be able to detect changes in ambient photoperiod to phase its secretion with the daily light/dark cycle.

Perhaps distinct nocturnal melatonin secretion begins only after birth when the newborn is first exposed to retinal light changes. Indeed, some (but not all) lambs exhibit a clear nocturnal melatonin rise by 1 week of age. For example, in studies of four sets of newborn twins (male/female) of the same age and weight, a circadian rhythm of melatonin was evident in both individuals of two pairs; however, in the other two pairs, only ambiguous patterns were found.[251] Because the differences in ontogeny of melatonin were greater between twin pairs than between siblings within a pair, there may be some genetic basis for the differential rate of development of circadian melatonin rhythmicity. By at least 6 weeks of age, nocturnal rises in melatonin are well established.[252] Moreover, the young lamb can shift its melatonin rhythm to accord with a new photoperiod based on the patterns of circulating melatonin in lambs and their mothers shifted from long days (short durations of daily melatonin secretion) to short days (long durations of daily melatonin secretion). As shown in Figure 31.20 (left), the daily period of increased melatonin, which is of short duration during long days, begins to expand during the first long night. By 2 weeks of age, the melatonin rhythm was entrained completely in all lambs; of importance, the rate of entrainment for the lambs was the same as for the mothers (Figure 31.20, right). Arguments that either melatonin may be transferred from the lactating mother through the milk or that the melatonin rhythm of the lamb is entrained through some other maternal mechanism are not likely to be tenable based upon considerations reviewed elsewhere.[252] Thus, in view of the foregoing data from the fetus and early postnatal lamb, the developing sheep receives information about ambient day length via the mother before birth and then via its own secretory profile of melatonin during the early postnatal period.

Surgical removal of the adult pineal gland abolishes the inductive reproductive response of the female to short days and the inhibitory response to long days.[219] However, an initial study of the role of the pineal gland in the onset of puberty in the female sheep was unable to detect any abnormal timing of first matings in pinealectomized lambs outdoors.[253] This surprising finding was attributed to the possible photoperiod insensitivity of the Merino, a breed of sheep with a relatively long annual breeding season and an indistinct anestrous period. A subsequent study from the same laboratory found that pinealectomy delayed puberty in crossbred

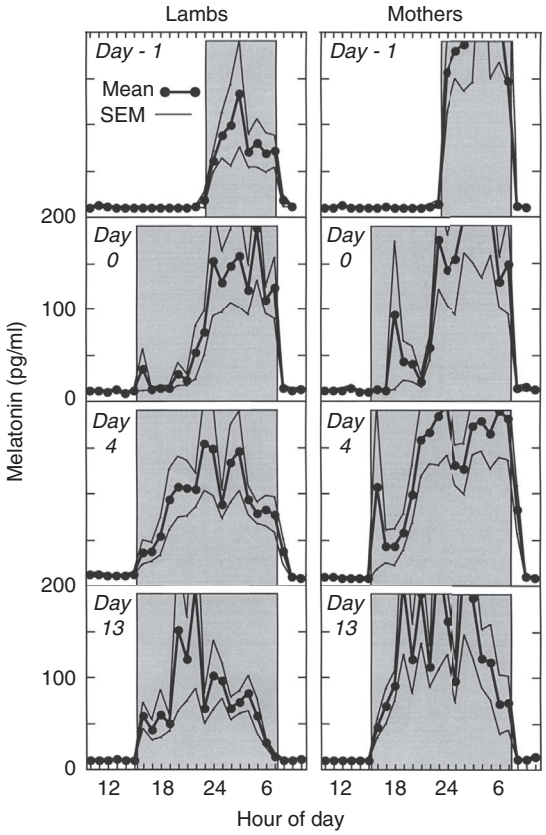

FIGURE 31.20 Change in melatonin pattern after shift from long days to short days. Circulating melatonin (mean ± SEM, $n = 8$) is shown during a 24-h period for 7-week-old lambs (*left*) and their mothers (*right*) on long days (Day −1), on the day of the shift to short days (Day 0), and on Days 4 and 13; periods of darkness (*shaded areas*). *From Ref. 252, with permission.*

Merino lambs (×Border Leicester × Dorset breeds)[254]; clearly, breed differences in the photoperiod modulation of puberty remain to be clarified for the sheep. Another approach to disruption of the pathway for transmission of photoperiod information is ablation of the sympathetic innervation of the pineal gland by bilateral removal of the superior cervical ganglia (see Figure 31.15 for pathway). After this procedure, melatonin secretion may continue, albeit at very low levels, and any modest rises are not consistently related to any particular time of the photocycle (Figure 31.21, bottom inset). Superior cervical ganglionectomy of lambs of the Suffolk breed at an early age (6 weeks) delays initiation of reproductive cycles, suggesting that normal pineal function is necessary for timing puberty to the appropriate season (Figure 31.21).[255] Cycles do begin in lambs in which pineal function is absent (pinealectomized[254]) or impaired (ganglionectomized[255]), but they are unusually late; such females may even attain puberty at the time when untreated lambs end their first breeding season and enter seasonal anestrus. From these studies and those using continuous long days or short days, the

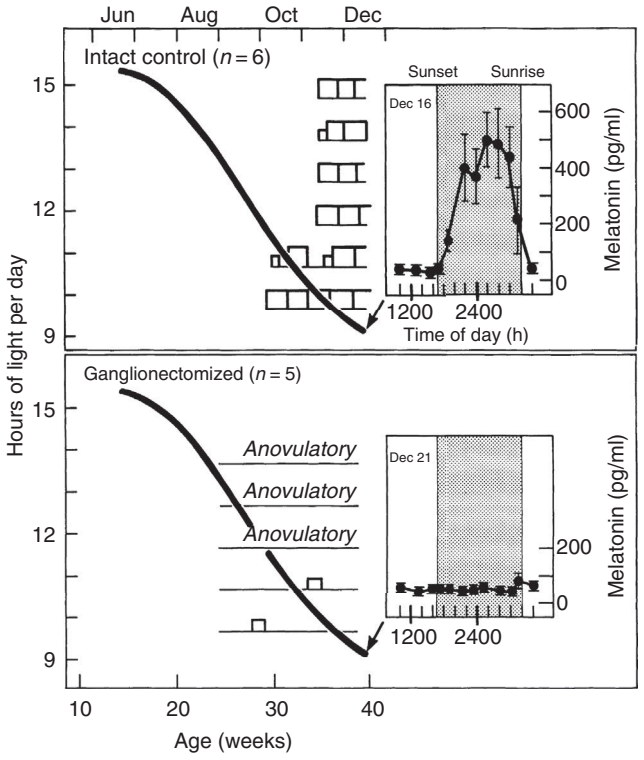

FIGURE 31.21 Denervation of the pineal gland of the lamb prevents onset of repetitive reproductive cycles at the normal age in natural environment (*bottom*). The 24-h pattern of circulating melatonin (mean ± SEM (insets)) is shown at 40 weeks in lambs in which the superior cervical ganglia were removed bilaterally at 6 weeks of age (*bottom*); also shown is the melatonin pattern in unoperated postpubertal lambs that were exhibiting normal reproductive cycles (*top*) (see Figure 31.19 for details of coding of luteal phases). *From Ref. 25, with permission.*

concept is reinforced that photoperiod cues can readily modify the timing of puberty, but they cannot forestall or advance puberty outside of certain limits. Finally, melatonin is involved in regulating the timing of puberty by photoperiod in the female sheep[254–257] because the pattern of melatonin encodes information about day length, as in the adult.

Interestingly, chronic administration of melatonin by implants, which is a relatively easy method to deliver the indolamine, has been used in an attempt to alter the onset of ovarian cycles in the lamb. Constant-release devices do not produce a circadian pattern of hormone, but as in the adult,[258,259] chronic melatonin treatment may be interpreted as a series of short days. In this regard, melatonin implants have been reported to have no effect, to advance puberty, or to delay puberty in pineal-intact lambs raised outdoors.[255–257] The varied effect appears to be related to the age when chronic treatment began; delayed puberty was associated with early initiation of melatonin administration, and precocious puberty was associated with a later insertion of implants. Similar results have been obtained for the timing of puberty and age of exposure to short days.[237] A great deal remains to be learned from such studies that attempt to use principles about photoperiod to time onset of reproductive cycles through practical approaches that do not require circadian delivery of melatonin (see Ref. 233 for discussion).

ONTOGENY OF PHOTOPERIODIC RESPONSES

It is clear that the young lamb can receive photoperiod information even before birth via the maternal melatonin rhythm. Can such information be used to influence the neuroendocrine control of hormone secretion? Thus far, studies have largely examined the regulation of prolactin in this context; even though this hormone has little to do with puberty in the sheep, prolactin is consistently increased by long days and decreased by short days in a steroid-independent manner. It is tempting to conclude that photoperiod can influence fetal neuroendocrine function from the observation that the level of fetal prolactin in late gestation differs according to time of year.[260] More direct evidence that photoperiod is the key seasonal factor regulating early prolactin secretion has been derived from a study using artificial photoperiod.[261] In that study, the results of which are shown in Figure 31.22, pregnant sheep were maintained in either short days (8L:16D) or long days (16L:8D) for the final third of gestation. Then, on the day of birth, all lambs and their mothers were transferred to an intermediate photoperiod (12L:12D), and prolactin was monitored daily. In lambs gestated in long days, circulating prolactin at birth was high, which most likely reflects a carryover of this pattern of secretion from before birth. By contrast, lambs gestated in short days had low concentrations of circulating prolactin at birth. During the neonatal period, the high levels of prolactin in long-day gestated lambs declined in the equatorial photoperiod, whereas in short-day gestated lambs, circulating prolactin increased in response to the 12L:12D photoperiod. It is noteworthy that there was no sex difference in patterns of circulating prolactin—a finding that is of general interest for the later discussion of differences in the timing of puberty in male and female lambs. The results of this study demonstrate two aspects of the fetal response to photoperiod cues received by the mother. First, photoperiod can modulate neuroendocrine functions before birth, thus accounting for the differential prolactin secretion at birth. Second, the lamb begins to construct a photoperiod history before birth, with which it can interpret photoperiod signals postnatally. Therefore, in the study illustrated, long-day gestated lambs respond to the 12L:12D photoperiod as a short day because it is a decrease relative to the photoperiodic signals received in utero; the short-day gestated lambs react as if this postnatal photoperiod were a long day.

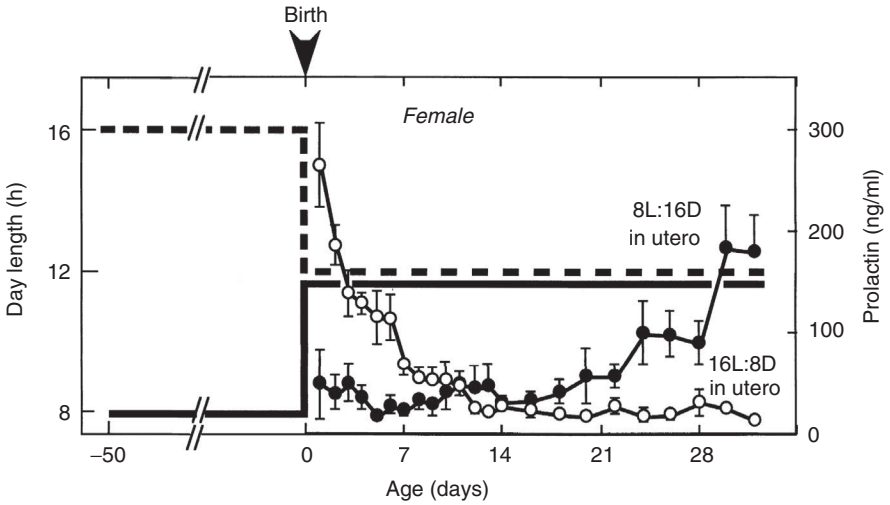

FIGURE 31.22 Circulating prolactin concentrations in female lambs in a 12L:12D, born to mothers maintained in a 16L:8D (*open circles*) or 8L:16D (*solid circles*) during late gestation. Values are mean ± SEM; $n = 7$. *From Ref. 262, with permission.*

GROWTH DURING DIFFERENT SEASONS AND TIMING OF PUBERTY

The photoneuroendocrine system of the developing female sheep transmits the main seasonal cue used to time the decrease in sensitivity to steroid feedback and permits the expression of the high-frequency GnRH pulses driving the pubertal follicular phase. However, this can only occur if growth cues related to energy metabolism indicate that physiologic size is appropriate to support the consequences of pregnancy and lactation.

This interaction between growth (and by inference, nutrition or energetic state) and environment is illustrated in Figure 31.23, which presents the time of initiation of reproductive cycles in groups of lambs that are born at the same time of year (spring), but for which the growth rates differ. The study[231] was conducted in natural photoperiod, with the rate of growth altered by changing the availability of food. Some females, well fed from birth, grew rapidly and puberty occurred at the usual age (Group A, ~30 weeks). Other lambs (Groups B, C, and D) were placed on a restricted diet for varying periods after weaning and before they were fed ad libitum. The onset of reproductive cycles was delayed by various amounts in such females. Despite their having experienced an appropriate photoperiod for initiation of ovulations, puberty did not occur when they were growth retarded, presumably because sufficient energy was not available to support the consequences of activation of the reproductive system. This underscores the importance of reaching a metabolic balance where somatic growth is sufficient, but where energy is also available for other processes such as reproduction. Photoperiod time measurement is maintained in growth-retarded lambs[263]; when they were then induced to grow during the autumn and winter breeding season (Groups B and C), cycles began at a smaller size (i.e., 35 kg) than for controls (i.e., 45 kg). This raises the possibility that the normally growing females (Group A) had grown sufficiently to have the potential to attain puberty earlier in the year (August), but day lengths were too long at the younger age (i.e., 25 weeks) to reduce hypersensitivity to estradiol feedback inhibition. Later in the year (October), when day length became much shorter, sensitivity to estradiol negative feedback could be reduced to initiate reproductive cycles (i.e., 30 weeks of age). Finally, when the phase of rapid growth was induced during the anestrous season (Group D), the lambs grew well beyond the normal size required to initiate reproductive cycles, but they remained anovulatory. These females maintained hypersensitivity to estradiol negative feedback because of the long days of summer, and it was not until the decreasing day lengths of autumn that the cycles could begin. Thus, both season and size serve as determinants timing the initiation of reproductive cycles in the lamb through photoperiod and growth-related cues. This same conclusion was reached earlier when season of birth, rather than growth, was experimentally altered (Figure 31.16).

It is of interest that photoperiod itself can modify growth rate (see Ref. 264 for a review). In lambs, increasing daily exposure to light increases both food intake and efficiency of amount of feed consumed to amount of weight gained. The hormonal mechanisms for the photoperiod-induced increases in growth remain to be established. The anabolic effect of long days does not appear to be associated with alterations in growth hormone, insulin, or thyroxine because these hormones are unaffected by photoperiod. Although pinealectomy prevents photoperiod-induced changes in prolactin and photoperiod-induced increases in growth rate in sheep,[265] there is no definite proof that prolactin is the hormonal mediator of increased growth under long days.

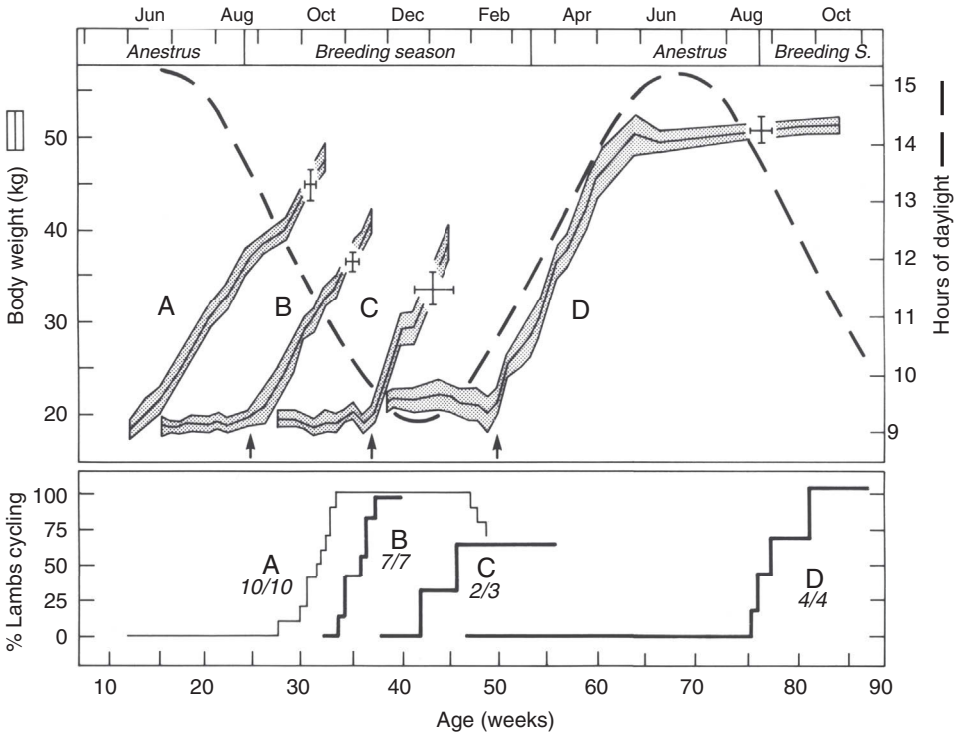

FIGURE 31.23 Season and growth influence timing of onset of reproductive cycles in female lambs (top, mean (±SEM) BW and age at first luteal phase; bottom, cumulative % ovulating for each group). Lambs were born in spring (March) and were raised outdoors (*broken line*, photoperiod). They were either fed ad libitum after weaning at 10 weeks of age (Group A), or were placed on a restricted diet of similar composition (Groups B, C, D); at various ages (*arrows*), feeding ad libitum was begun in food-restricted lambs. *From Ref. 231, with permission.*

Sexual Maturity vs Puberty vs Onset of Breeding Season

In a developing seasonal breeder, it is difficult to distinguish among the terms "sexual maturity," "puberty," and "onset of breeding season." Puberty in the young female and onset of breeding activity in the adult after seasonal anestrus are both transitions from anovulatory conditions to those of cyclic ovarian function. An increase in GnRH pulse frequency is considered to underlie each transition.[39,109] Because of this, the hypothalamus can be viewed as the ultimate common final pathway governing the two states of reproductive activity (see Refs 266 and 267 for reviews). The rhythm of the GnRH neurosecretory system is modified by estradiol such that during periods of high feedback sensitivity, its activity is slowed. The female lamb is born exquisitely sensitive to estradiol negative feedback. The reduction in sensitivity first occurs at puberty (Figure 31.6) and then occurs at each transition from the anestrous to the breeding season. Thus, the ability to alter feedback sensitivity, first used by the lamb as a mechanism to regulate the tempo of sexual maturation, is retained during adulthood to regulate seasonal reproduction.

Because the developing sheep uses environmental cues to time the first cycles (puberty) to the appropriate season for conception (breeding season), a key question becomes, "When does *sexual maturity* occur?" To provide an appropriate setting to begin to answer this question, it is instructive to return to the case of the autumn-born lamb and the timing of its first reproductive activity (Figure 31.16). Recall that such lambs remain anovulatory during the summer anestrous season despite having attained the appropriate age and size for initiation of reproductive activity. Then, several months later at the onset of the adult breeding season, they initiate ovulations without further growth. There are two conceptually different explanations to account for this delay in the autumn-born lamb: (1) development is retarded by inhibitory photoperiod (long days), and the delayed onset of cyclicity reflects delayed sexual maturity; (2) development is not retarded but long-day photoperiod induces seasonal anestrus at sexual maturity, and in this case, the onset of cyclicity simply reflects the onset of a breeding season when day length decreases sufficiently. According to the latter explanation, the transition to adulthood is "masked." If this argument were valid, then even in the normal spring-born lamb sexual maturity is masked. Interestingly, puberty in spring-born lambs occurs at a later time than onset of the breeding season in adults.[41,106] Although the underlying reason for this is unclear, it may involve a differing requirement in the shortness of days to induce the change in estradiol negative feedback in adult females versus lambs. Such an argument for masking by photoperiod has been inferred from the results found in Figure 31.23 where rapidly growing lambs (Group A) initiated reproductive cycles at a size (~45 kg) much larger than that required for this phenomenon (~30–35 kg). According to this explanation, they continued to grow and remained anovulatory until day length became shorter. Support

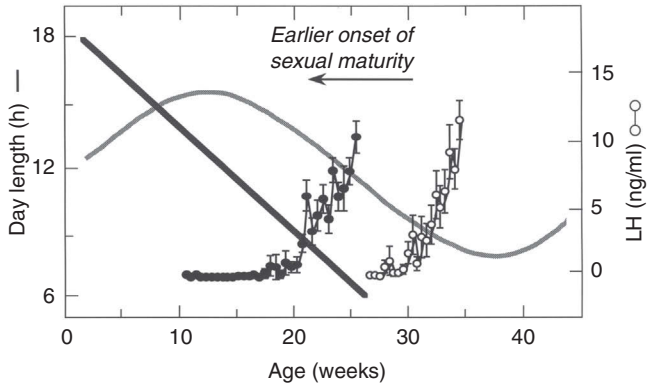

FIGURE 31.24 Increase in mean (± SEM) circulating LH in rapidly-growing estradiol-treated, ovariectomized lambs exposed to either natural photoperiod (*open circles*, $n=7$) or to decreasing artificial photoperiod (*solid circles*, $n=8$) from birth. *Artificial photoperiod data from Ref. 268.*

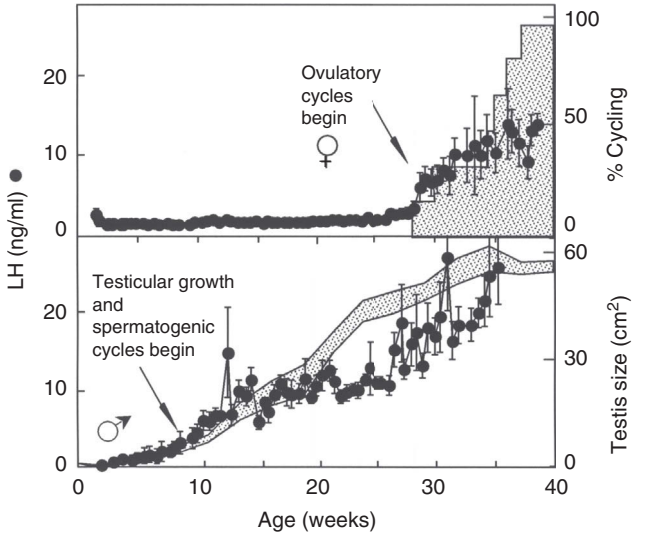

FIGURE 31.25 Sex differences in the timing of the pubertal increase in GnRH secretion and gonadal activation. Mean (± SEM) LH concentrations (*solid circles*) in gonadectomized female (*top*) and male (*bottom*) sheep treated chronically with a constant level of estradiol by subcutaneous Silastic implant. The time of the increase in LH secretion is associated with the onset of ovulatory cycles (shaded histogram) in intact females, and testicular growth and spermatogenesis (shaded line) in intact males. *Redrawn from Ref. 270.*

for this has been obtained by simply exposing lambs earlier in life to a permissive (stimulatory) photoperiod that markedly advances initiation of reproductive activity to a younger age and smaller size[268] (Figure 31.24). In the example shown, exposure of females to a gradually decreasing artificial day length beginning at birth allowed reproductive activity to be initiated at 20 weeks of age. Typically, puberty occurs at 30 weeks of age outdoors when day length begins to decrease at approximately 15 weeks of age. Thus, advancing the decrease in day length by 15 weeks, advanced puberty by 10 weeks. By using such approaches, the minimal age for the onset of reproductive activity as timed by growth cues should eventually be revealed. This knowledge is essential to establish when growth cues serve as important internal determinants of somatic size timing onset of reproductive activity and when photoperiod cues serve as an important external determinant synchronizing the expression of sexual maturity with the appropriate season. Only then will we be able to rid ourselves of the confusing terminologies presently used and be able to explain how reproductive activity is timed in more conceptual terms. Until then, it would appear that "sexual maturity" of the female (potential to ovulate) is attained well before "puberty" (onset of ovulations).

SEX DIFFERENCES IN TIMING THE TRANSITION INTO ADULTHOOD

There is a profound sex difference in the timing of puberty in many species. In the sheep, spring-born male lambs begin reproductive development at about 10 weeks of age in midsummer, as evidenced by the onset of spermatogenesis,[56,269] whereas females remain prepubertal until 25–35 weeks in the autumn when they first ovulate (Figure 31.25). This strategy maximizes the reproductive opportunity for each sex. It is reasonable that the young male initiates the pubertal process earlier. A longer time is required for spermatogenesis (5–10 weeks) than for the development of an early antral follicle to the preovulatory stage (few days). More importantly, the prolonged exposure to progressively increasing levels of testosterone is necessary to continue to program and reinforce behaviors necessary for competition with older males and mating with females. Such a reproductive strategy would be particularly relevant in species where the initial matings and conception occur in the first autumn after birth. By contrast, very fast maturing, short-lived species (i.e., rodents) that first mate within weeks after birth in spring and summer may not be expected to exhibit such pronounced differences in the age at puberty. Similarly, in long-lived species that mature over an extended time (years), early puberty in the male would have no obvious advantage. In fact, males of higher primate species, such as the rhesus monkey and the human being, mature at an even slower rate than females.

Sex Differences in the Pubertal Increase in GnRH Pulse Frequency

Studies of the underlying cause of the sex difference in initiation of puberty face the obvious problem of how to compare reproductive development between the two sexes. In most species, the outward signs of puberty in males and females are different and are therefore difficult to equate. These physical and behavioral changes

during puberty are produced by sex steroid hormones, but differences in quality, quantity, and pattern of steroids arise from differences in the production of the male and female gametes (continuous liberation of spermatozoa vs the cyclic release of ova). Common to both sexes, however, is the antecedent rise in LH (GnRH) secretion during puberty when the sensitivity to gonadal steroid feedback inhibition decreases. This phenomenon has been demonstrated in both sexes (Figure 31.25). Therefore, studies on sexual differentiation of the timing of puberty have used the pubertal increase in LH (GnRH) secretion as an index of "neuroendocrine sexual maturity" because it allows comparison of sex differences in the development of control systems within the brain unencumbered by differences in the gonad (ovary vs testis). In this "neuroendocrine" model (neonatally gonadectomized lamb replaced with a constant feedback of exogenous steroids), the pubertal increase in LH is robust in both sexes. It positively correlates with indices of sexual maturation (initiation of testicular maturation and ovarian cycles) in gonad-intact males and females. The sustained increase in LH secretion beginning shortly after birth in the male indicates that a decrease in sensitivity to steroid inhibitory feedback occurs much earlier than it does in the female. The model uses estradiol as the steroid inhibitory feedback hormone in both females (to replace estradiol secreted by the ovary) and males (to replace estradiol produced by aromatization of testosterone in the brain).[271–273] Although testosterone is the major sex steroid produced by the testes, previous work showed that puberty-related changes in LH secretion in intact male lambs could be equally recapitulated in castrated male lambs implanted with either testosterone or estradiol,[271] suggesting that, similar to females, estradiol may be the major inhibitor of GnRH/LH release in prepubertal males. Nonetheless, a role for the actions of testosterone per se or its metabolite, dihydrotestosterone, cannot be ruled out and has not been examined to date in prepubertal male sheep. In yearling male sheep, blockade of 5α-reductase, the enzyme that synthesizes dihydrotestosterone from testosterone, increased pulsatile LH secretion in testosterone-treated castrates,[274] suggesting that dihydrotestosterone may mediate, at least in part, inhibition of GnRH release by testosterone in young adult males.

Programming of Sex Differences in the Timing of the Pubertal GnRH Rise by Prenatal Steroids

The pubertal increase in LH secretion in the male is programmed before birth through the organizing action of testicular steroids on the brain mechanisms controlling sensitivity to the feedback action of estrogen. This inference was made because prenatal exposure of the female fetus to exogenous testosterone advances the age when high-frequency LH pulses are expressed (neuroendocrine sexual maturity) using the neuroendocrine model (estradiol implant, no ovaries). In the study illustrated in Figure 31.26(A)–(C) using the neuroendocrine model, testosterone was administered to pregnant sheep and as expected, this treatment masculinized the external genitalia of female lambs.[56] Importantly, the pubertal increase in circulating LH in these developing females occurred at the same time as that in males which was several months earlier than normal females. In this study, more frequent measurements provide evidence that this precocious increase in LH is due to an increase in LH pulse frequency, not amplitude. Reduced sensitivity to estradiol negative feedback before onset of puberty was confirmed in a later study.[275] The *time of testosterone exposure* during development is important, with the critical period in early pregnancy. Exposure of the unborn female between Days 30 and 90 of gestation (~150 days term) produces the profound advance in the timing of the pubertal GnRH rise, whereas the same treatment from Days 89–135 of pregnancy is without effect (data not shown, see Ref. 56). The *duration of*

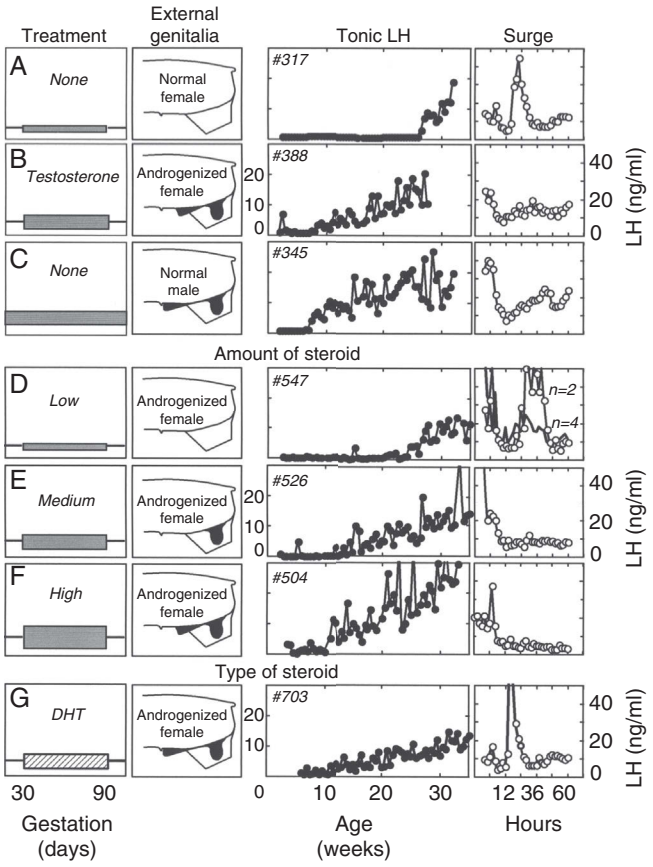

FIGURE 31.26 Prenatal exposure to testosterone (*panels a-f*) or DHT (*panel g*) and its effect on external genitalia (*left*), the developmental pattern of tonic LH secretion (*middle*), and the LH surge (*right*). The lambs were gonadectomized within 3 weeks after birth and implanted with a Silastic capsule containing estradiol. *Redrawn from Ref. 270.*

testosterone exposure is important. Treatments for a short duration (e.g., Days 30–60 or Days 60–90) are effective, but the timing of the pubertal LH rise is less advanced (data not shown, see Refs 276 and 277). The *amount of testosterone exposure* is important as determined in a dose–response study (Figure 31.26(D)–(F)).[278] The patterns of LH secretion were related to the amount of testosterone experienced as assessed by the external genitalia. Those treatments producing the greatest external masculinization also produced the earliest decreases in response to steroid feedback and, hence, the earliest increase in LH secretion. The *type of steroid* is important. Because testosterone can be converted to a more potent androgen or an estrogen by the brain, the type of steroid action could not be determined in studies in which testosterone alone was administered. Administration of dihydrotestosterone, the androgenic metabolite that cannot be converted to an estrogen, also advanced the time of the pubertal rise in LH (Figure 31.26(G)) suggesting that androgenic action is involved in this programming.[279] Although this pure androgen produced similar results as testosterone, they were not identical, leaving some possibility for a role of estrogens in organizing the timing of the pubertal increase in GnRH secretion.

Masculinization of the timing of puberty most likely occurs in the neural control of GnRH secretion because the effect of prenatal androgens was to increase the frequency, rather than the amplitude of LH pulses. Androgen effects on pituitary function are less likely as the pituitary response to GnRH challenge is neither affected by androgenization nor is it sexually differentiated in the sheep.[280,281] However, Robinson et al.[282] recently reported that prenatal androgenization with testosterone increased pituitary weights in both male and female offspring by 40%. This effect was not seen in lambs treated in utero with dihydrotestosterone, suggesting that the effect was due to aromatization of testosterone to estrogen. In addition, testosterone decreased the amount of colocalization observed for LHβ and ERα, perhaps indicative of a decreased ability to respond to estradiol-negative feedback that may explain, in part, the hypersecretion of LH observed in this animal model.

Programming of Sex Differences in Other GnRH Controls by Prenatal Steroids

Besides organizing the timing of the pubertal increase in GnRH secretion by modifying the sensitivity to estradiol negative feedback, prenatal steroids also program brain sex differences in the function of other steroid feedback controls. This would be expected because neuroendocrine mechanisms timing the discontinuous liberation of ova are more complex than those for the continuous production of spermatozoa. Such differences in regulation are manifest during puberty when gametes are first produced and liberated. Indeed, in the sheep the neuroendocrine regulation of ovulatory cycles requires four well-known steroid feedback mechanisms to control GnRH secretion. Two feedbacks regulate the surge mode of GnRH secretion—the stimulatory feedback action of estrogens to induce a GnRH surge and the ability of progesterone to block this stimulatory feedback action; two feedbacks control the pulsatile mode—the inhibitory feedback of estrogens and the inhibitory feedback of progesterone. In the male, three of these mechanisms are not necessary; for central regulation of the control of spermatogenesis, only the simple, estrogen negative feedback system is required. To explain how these differences arise, one must consider whether the female sheep adds the extra feedback control systems or the male sheep removes them. The latter is the most salient explanation. According to this hypothesis, multiple feedbacks are inherent in both genetic sexes, and they require no further developmental input for normal function during adulthood. However, if the ovine fetus is male, the three irrelevant steroid feedback controls of GnRH secretion are either abolished or become functionally inoperative through programmed reductions in sensitivity. This central programming in the ovine male occurs through exposure of the developing brain to his own testicular steroids that act as androgens and estrogens to organize the brain during a critical period of development. In the sheep, neuroendocrine programming begins before birth and continues well into postnatal life. Evidence for this scheme is accumulating as shown by exposure of developing lambs to combinations of exogenous testosterone and estradiol (Refs 283 and 284; for reviews, see Refs 285–288).

GnRH Surge System

The GnRH surge system is not functional in the male. Prenatal treatment with testosterone perturbs the ability to respond to the stimulatory feedback action of estrogen in young adults (high doses abolish the response; low doses prolong the latent period). Whether the failure of the LH surge to occur in the normal male or in females prenatally treated with testosterone is due to deficits in GnRH release has been tested. GnRH was measured in the pituitary portal system during standardized estradiol treatment that induces preovulatory surges of the neurohormone in normal females.[289] A prolonged discharge of GnRH was not detected in individuals prenatally exposed to endogenous or exogenous testosterone, indicating that indeed the early programming of the GnRH surge mechanism is at the level of the brain. Whereas exposure to high doses of testosterone abolishes the surge mechanism (Figure 31.26(E) and (F), right), low doses do not (Figure 31.26(D), right).[278] In low-dose females, the surge system is retained during adulthood, but the latent period from the administration

of estradiol to the LH surge is prolonged. There may also be some effect of breed as ovary-intact Suffolk sheep treated from days 30–90 twice weekly before birth with either 200 mg[290] or 100 mg[291] testosterone propionate displayed delayed surges that were reduced in amplitude, but ovary-intact Polled Dorset sheep similarly treated prenatally with testosterone propionate[292] exhibited a total absence of LH surges in response to estradiol. Prenatal administration of dihydrotestosterone—the potent, nonaromatizable androgen—has no effect on the GnRH surge system (Figure 31.26(G), right)[279] suggesting that the programming of the GnRH surge mechanism is due to the estrogenic action of testosterone. This is of interest because prenatal treatment of the female with dihydrotestosterone reduces the sensitivity to the negative feedback action of estrogen early in life and results in a precocious pubertal rise in LH secretion (Figure 31.26(G)). The androgenic action of testosterone on timing the pubertal increase in GnRH has been further confirmed by blocking the androgen receptor with the antagonist flutamide.[293] Thus, the tonic and surge modes of GnRH secretion may be organized by the two different metabolites of testosterone. Perhaps prenatal androgens organize the level of sensitivity to the inhibitory feedback action of steroids and the timing of the pubertal GnRH rise, whereas prenatal estrogens organize the mechanisms involved with the stimulatory feedback action of estrogen and production of the GnRH surge.

Progesterone Feedback Systems

Two progesterone feedback controls of GnRH exist in the female sheep. One regulates the surge mode of secretion as, during the ovulatory cycle, progesterone can block the stimulatory feedback action of estrogen to prevent the GnRH surge. Whether this regulatory system is sexually differentiated is not known because the blocking actions of progesterone cannot be examined in males or females in which the surge system has been masculinized (i.e., by exogenous testosterone or estrogen). An androgenic action might masculinize this action of progesterone, but this possibility has not been tested; if this occurred, it would suggest that the sexual differentiation of GnRH feedback controls is more complex than presently envisaged. The second feedback action of progesterone is clearly sexually differentiated, namely the inhibition of tonic GnRH secretion to prevent high-frequency GnRH secretion from occurring during the luteal phase. In females treated with testosterone before birth, LH pulse frequency is greater than in normal females even though circulating progesterone concentrations are similar.[291] This was predictable because when this inhibitory feedback action of progesterone was tested earlier in both sexes in the absence of endogenous gonadal steroids, the male sheep was much less responsive than the female; prenatal treatment of females with testosterone reduced the response.[294,295] Cheng et al.[296] reported that prenatal treatment of sheep with testosterone reduced the number of dynorphin-immunopositive cells in the arcuate nucleus while having no significant effect on the number of kisspeptin-positive cells. As dynorphin in this area has been implicated to mediate progesterone feedback inhibition[85–87] and kisspeptin stimulates GnRH/LH release, perhaps prenatal exposure to testosterone programs a change in the balance between stimulatory and inhibitory input to reduce the postnatal response to progesterone feedback inhibition of GnRH secretion.

It will be of interest to understand how independent the mechanisms responsible for development of the tonic mode of GnRH secretion are from the surge mode. In addition to differences in the type of steroid involved in each, there may also be differences in critical periods. As originally conceived from studies of the differentiation of the surge mode, the "critical period for sexual differentiation" denotes the broad period of sensitivity to organization by gonadal steroids (see Ref. 297 for a review). An emerging concept is that the "critical period" is not one single entity. So far in the sheep, discrete critical periods for the control of anatomy, behavior, and neuroendocrine function have already been noted.[298–300] There is additional evidence that postnatal exposure to estradiol may be important for complete defeminization (or masculinization) of the surge mechanism. Ovary-intact lambs treated prenatally with testosterone did not exhibit LH surges in response to estrogen.[284] However, lambs in that study that were treated prenatally with testosterone, but ovariectomized soon after birth, did exhibit LH surges, albeit they were longer in duration and appeared to be of a decreased magnitude. Thus, each sexually dimorphic trait may have unique requirements for its sexual expression regarding the duration or type (androgen vs estrogen) of steroid exposure. We already have evidence to support this possibility based on the different organizational actions of androgens and estrogens on the feedback mechanisms controlling the tonic and surge modes of GnRH secretion.

Endocrine Disruptors: A Case for Fetal Origin of Reproductive Dysfunctions

Given the sensitivity of the reproductive axis to both the organizational and activational effects of steroids, it is not surprising that the effects of endocrine disrupting compounds on reproduction has been the focus of much research in recent years. Data examining the effects of such compounds on reproduction in ruminants are limited largely to examining the effects of bisphenol A and octylphenol (both estrogenic in nature), polychlorinated biphenyls (PCBs; estrogenic, androgenic, antiestrogenic and antiandrogenic activities), and methoxychlor (estrogenic, androgenic, and antiandrogenic activities)

(for a review, see Ref. 301). The Environmental Protection Agency has identified hundreds of compounds that are exogenous in nature, but which can have significant effects on the structure or function of the endocrine system. Such compounds can be found in manufactured consumer goods, but also exist as plant-derived materials, such as the phytoestrogens. Many of these chemicals can bind to steroid receptors. The sheer number of compounds and a potential for their effects to be compound-specific and dependent upon factors such as dose, time of exposure, route of exposure, and differing periods of sensitivity during development have made their study difficult. Further, studies addressing the influence of endocrine disrupting chemicals usually involve acute exposure to relatively high doses, whereas exposure under normal circumstances usually involves chronic exposure to low doses or exposure to low doses of several chemicals at one time which may be synergistic or additive. Nonetheless, data is developing in ruminants to suggest that endocrine disrupting compounds could significantly affect reproduction at all levels of the hypothalamic-pituitary-gonadal axis.[302] Administration of bisphenol A from day 30 to day 90 of gestation in sheep increased LH secretion during the first postnatal month of life and decreased the amplitude of the postpubertal LH surge[303] while treatment with methoxychlor for the same period did not influence LH but delayed the LH surge. A subsequent study by this group examined both stimulatory and inhibitory actions of exogenous steroids and concluded that the defects seen in the earlier work was due to ovarian deficits as estradiol stimulatory feedback and inhibitory feedback by either progesterone or estradiol appeared normal.[304] A study by Evans et al.[305] reported that bisphenol A administration for 5 weeks beginning at 4 weeks of age in female lambs reduced the increase in LH pulse frequency normally seen in response to ovariectomy. In goats, administration of valproate, an antiepileptic drug thought to have effects both centrally on LH secretion and on gonadal conversion of testosterone to estrogen, from two to 10 months of age reduced LH secretion,[306] as did administration of PCB153, but not PB126, during gestation.[307,308]

Only a very limited number of studies have examined the timing of puberty in ruminants following exposure to endocrine disrupting chemicals. Puberty onset was accelerated in female lambs exposed to octylphenol from day 70 of gestation to weaning, day 70 of gestation to birth, or from birth to weaning.[309] Interestingly, administration of octylphenol after weaning did not appear to influence LH secretion,[305] raising the possibility that windows of sensitivity to this disruptor exist. Savabieasfahanbi et al.[303] reported that puberty was unaffected by bisphenol A or methoxychlor administration during gestation, even though both influenced amplitude or timing of the LH surge and bisphenol A caused an early cessation of the breeding season. In goats, puberty onset was delayed following exposure of females to valproate from 2 months to 10 months of age,[306] and in both male[307] and female[308] goats following PCB153 exposure. The delay in puberty observed in male goats was associated with increased DNA damage in sperm.

The mechanisms whereby puberty may be affected by endocrine disruption are unclear, but may involve alterations in hypothalamic neuropeptide expression. Bellingham and coworkers[310] reported that maternal exposure to sewage sludge, a not totally characterized mixture of many anthropogenic endocrine disrupting compounds, decreased *KISS1* mRNA expression in 110 day old fetal hypothalamic tissue. A subsequent study by this same group using a similar approach reported a reduction in GnRH mRNA, GnRH receptor mRNA, and galanin receptor mRNA expression in fetal hypothalami.[311] The study by Bellingham et al.[310] also observed a decrease in kisspeptin, LH-β subunit, and estrogen receptor-α protein expression (as measured by immunocytochemistry) in fetal pituitary tissue, suggesting that the effects of sewage sludge may not just be localized to the brain. Thus, exposure in utero to either naturally occurring or man-made compounds that mimic endogenous endocrine activity have the distinct potential to influence subsequent reproductive events in both males and females.

Sex Differences in Response to Internal and External Factors Timing Puberty

Two major physiologic determinants timing the pubertal LH (GnRH) rise in the female sheep are growth and photoperiod. The response to one or both of these must be sexually differentiated because male lambs attain puberty at a smaller body size and under longer photoperiods (spring/summer) compared to female lambs (autumn). Moreover, the prenatally androgenized female lamb also exhibits puberty at a smaller size and longer day length. As indicated in the next two sections, the current evidence implicates a sexual differentiation of the photoperiod response mechanism, rather than those systems responding to growth-related cues. This leads to the hypothesis that testosterone emanating from the fetal testes acts within the brain during the critical period of development before birth to alter the reproductive response to photoperiod history.

Sex Differences in Response to Photoperiod

As discussed earlier, in the female sheep the initiation of reproductive cycles is not normally timed by growth cues, but instead, reproductive maturation is expressed in response to photoperiod cues. Much less is known about the role of photoperiod and maturation in the

male. Studies conducted in several breeds (Ile de France, Merino-Border Leicester, Suffolk) lead to the conclusion that the developing male sheep may be relatively insensitive to photoperiod.[256,312,313] This is illustrated by raising males and females in an artificial photoperiod simulating that which occurs naturally after birth in the spring (Figure 31.27, top) or in an artificially reversed photoperiod simulating birth in the autumn (Figure 31.27, bottom).[314,315] As expected in the natural simulate photoperiod, the pubertal LH rise in females began between the usual ages of 25 and 30 weeks, but in the reversed photoperiod, this was delayed beyond the end of the study. In males, the pubertal LH rise began at the same time in either photoperiod. In androgenized females, prenatal exposure to testosterone not only prevented the delay in the pubertal LH rise that occurred in the female in an inappropriate photoperiod, but advanced it to a much earlier age, one similar to that of the male. In the context of the hypothesis for sexual differentiation of the timing of puberty by photoperiod, these findings indicate that the default mechanism is to delay expression of sexual maturation during increasing or long photoperiods of spring and summer. Without prenatal programming by testosterone, as in the case of the normally developing female, breeding in these seasons will delay the initiation of pregnancy until the decreasing day lengths of autumn. As noted earlier, this strategy results in spring-born young. However, as in the normal male, exposure to fetal testosterone during a critical period of sexual differentiation alters the photoperiodic timing mechanism such that reproductive activity will begin regardless of day length when growth cues are appropriate. Interestingly, the male does not remain unresponsive to photoperiod. By the second year or earlier, the control of GnRH begins to respond to this seasonal cue as an adult, and reproductive activity waxes and wanes becoming locked into seasons of increasing and decreasing fertility. Although fertility is reduced but not lost in males during the nonbreeding season in most breeds, in some primitive breeds, such as the Soay sheep, the testes regress markedly during the non-breeding season and become aspermic. Thus, the male is programmed for a temporary hiatus in photoperiod control of its reproductive system during its formative stages in the transition into adulthood.

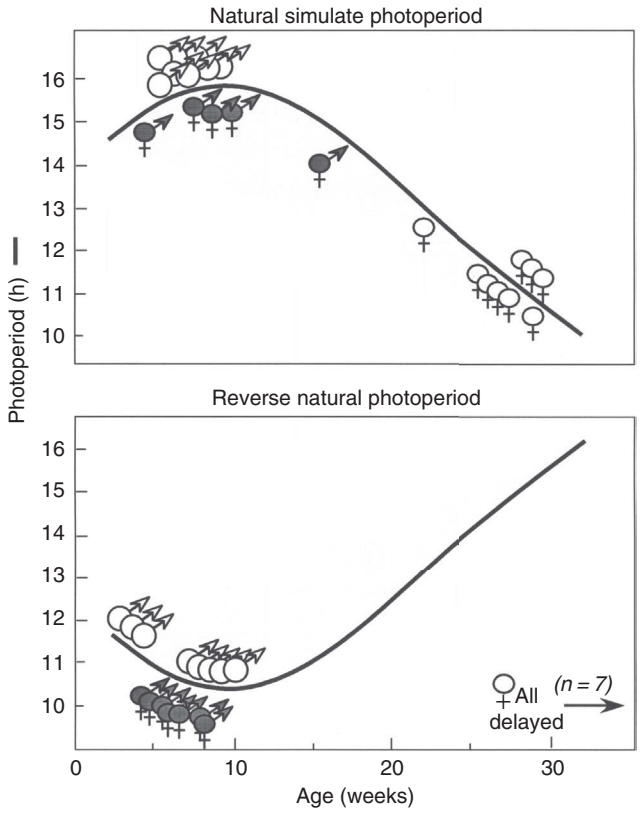

FIGURE 31.27 Photoperiodic regulation of the timing of puberty in male, female, and prenatally testosterone-treated female (shaded symbols) sheep. Symbols indicate timing of pubertal LH rise. *Top*: Animals raised indoors in a simulated natural photoperiod. *Bottom*: Animals raised indoors in a reverse natural photoperiod. Puberty was delayed in females in the reverse natural photoperiod beyond the end of the study. *Redrawn from Ref. 270.*

The sex difference in response to photoperiod is not due to a difference in the pattern of melatonin secretion. The pattern of melatonin is similar among males and females during development[251] indicating that the marked difference in timing of puberty in males and females does not originate from the pineal gland. Rather, it arises from a post-pineal difference in the mechanism by which the melatonin signal is subsequently used to govern the expression of sexual maturation. This type of reproductive development where the timing of puberty in the male is not influenced by photoperiod differs from the general developmental strategy for long-day breeding species. For example, in the short-lived grasshopper mouse[316] and field vole,[317] both sexes are responsive to photoperiod before puberty such that reproductive development is advanced by long days and retarded by short days.

Sex Differences in Response to Growth Cues

Bronson[318] has proposed that the growth requirements for activation of the reproductive neuroendocrine axis of the female should be more critical than those for the male because of her greater metabolic demands for reproduction (i.e., pregnancy and lactation). In view of this hypothesis, one could argue that the developing female would be more sensitive than the male to perturbations in metabolic signals and that such a difference might be revealed during growth restriction. A study comparing male and female developing sheep has determined if LH(GnRH) secretion is altered differentially by chronic growth retardation and acute increases and decreases in nutrition.[319] The gonads were removed to avoid the

confounding influence of gonadal steroids in developing males and females. No sex difference in gonadotropin secretion under chronic growth restriction was apparent, and LH pulse frequency became depressed equally in males and females during long-term malnutrition. For the acute responses, a small sex difference occurred—one in which males were somewhat more responsive than females to increases and decreases in level of nutrition on gonadotropin secretion. The results of these studies modulating energy intake indicate that the reproductive neuroendocrine system in the developing male is at least as sensitive, and perhaps even more so, than that of the female to changes in level of nutrition. Such findings do not support the notion that the growth requirements for the neuroendocrine control of puberty are markedly sexually differentiated.

CONCLUSION

Figure 31.28 summarizes and synthesizes an integrated model for puberty in the sheep by focusing on the GnRH neurosecretory system and the type of information that is integrated to increase the frequency of GnRH pulses. This model, although far from complete, accounts for both the sex difference in when this transition occurs and the different signals that time it in males and females. The core pattern of GnRH secretion in the developing sheep, like other long-lived species, is an increase in activity, and then a decline of varying durations followed by the pubertal rise. The response to the early increase in GnRH secretion that occurs before birth in the sheep, a precocial species, programs the type of information that is used to produce the sex-specific pattern of GnRH secretion after birth. If no testes are present in utero, multiple reproductive neuroendocrine feedback controls remain operative, and the (female) lamb becomes photoperiodic with respect to the timing of puberty using heightened sensitivity to steroid negative feedback. Although metabolic cues provide information that sufficient growth has occurred to begin reproduction, the young female cannot express her sexual maturity because the photoperiod is not appropriate. Sexual maturity is masked, the ovary remains quiescent, and she immediately enters seasonal anestrus. Once she has experienced the requisite photoperiod sequence, the long days of summer followed by the short days of autumn, the neuroendocrine sensitivity to steroid negative feedback decreases, and high-frequency GnRH pulses are expressed. The other feedback controls remain operative, and repeated ovulatory cycles are initiated to begin the breeding season. This strategy allows young females to synchronize the beginning of reproductive activity with those of mature, seasonally breeding females.

By contrast, the male lamb tracks photoperiod in utero much like the female lamb, but he does not use photoperiod to time the expression of high-frequency GnRH pulses. In response to the first increases in GnRH

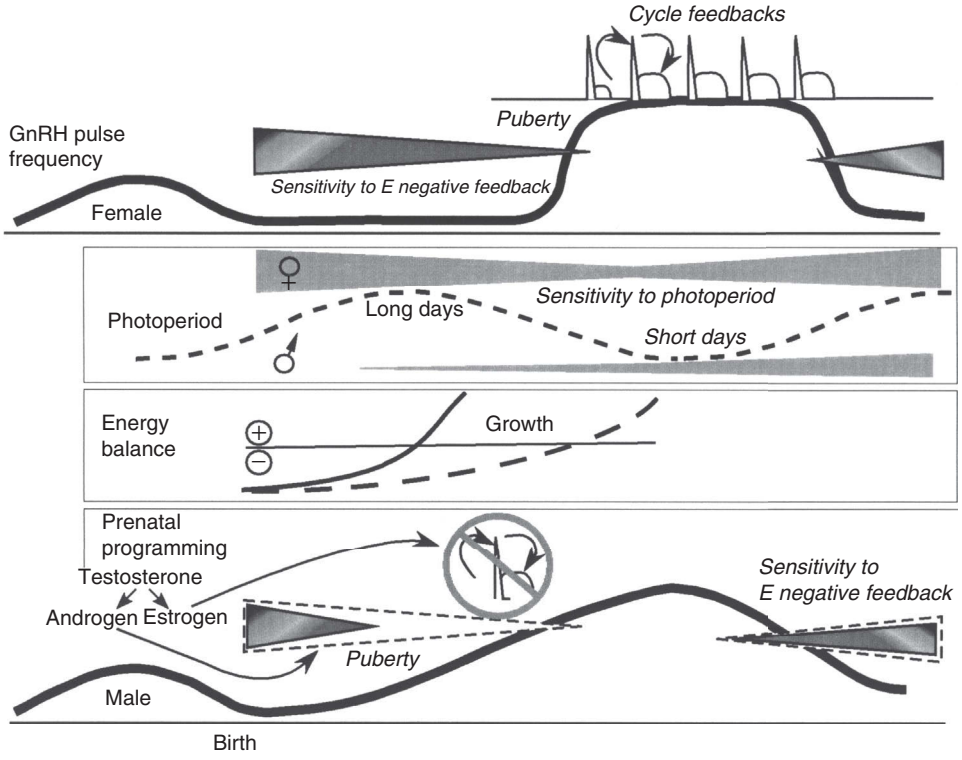

FIGURE 31.28 Integrated model for internal and external determinants timing the onset of puberty in the sheep.

secretion and the resultant gonadotropin drive to the testes before birth, testosterone is secreted to masculinize the photoneuroendocrine system; this effectively reduces the reproductive response to day length. Rather than considered to be masculinization, this programming could be thought of as defeminization because the testicular steroid action modifies an innate function. This is much like the defeminization of the unnecessary feedback controls in the male that underlie ovulatory cycles. By removing the photoperiod requirement for the expression of high-frequency GnRH pulses, puberty in the male begins once metabolic signals provide information that positive energy balance is sufficient for reproductive activity. Most interestingly, after further maturation, in the second year the reproductive neuroendocrine system of the young male does become regulated by photoperiodic cues, much like that of the female, to result in seasonal changes in sensitivity to negative feedback regulation of GnRH secretion. Thus, while metabolic cues time hypothalamic sexual maturity in both sexes, the presence or absence of prenatal programming by testosterone and its metabolites determines whether photoperiod serves as a permissive cue to begin reproductive activity. Viewed in a broad sense, different signals time "puberty" in the two sexes. Metabolic signals time puberty in the male, but photoperiod cues time puberty in the female. Perhaps the hiatus in photoperiodic control in the male during the first year is necessary to increase the GnRH/LH drive to the testes early to develop the behavior necessary to begin competition with older males for females. This strategy for sex differences in timing of puberty is a prime example of how fundamental mechanisms have been conserved while speciation has led to adaptive modification of the control of these mechanisms to allow for optimal timing of the onset of fertility.

The model described is far from complete. The terms puberty, sexual maturity, and initiation of reproductive activity are imprecise; fundamental questions remain to be asked and answered. So far we have focused on the simpler ones. This leads to consideration of ultimate and proximate signals, sensors, and pathways. There is little argument that an increase in GnRH secretion is the ultimate neuroendocrine signal that initiates puberty. This is not a unique signal, however, because such an increase also occurs at other transitions from infertility to fertility later in life. Similarly, understanding details of the *proximal* regulators (neurotransmitters) of GnRH is important, but it will not contribute to our knowledge of how puberty is timed. In addition, a deeper understanding of other proximal signals that are permissive such as photoperiod, social cues, and energy balance will also not help our understanding of what and where is the deep, unique proximal signal(s) that starts the cascade. The signaling mechanisms by which photoperiod influences puberty onset in females are now well understood, but their specific actions in the hypothalamus need to be identified. Our understanding of the metabolic signals that potentially provide information to the brain about energy availability is expanding. However, a number of questions remain unanswered about the signals, sensors, and pathways by which growth cues time puberty. For example, what specific aspect of metabolism or growth provides information to the brain that sufficient energy is available to begin reproductive activities that are induced by GnRH secretion? Is there one ultimate signal or is some combination of metabolic cues required? What specific brain regions/neural mechanisms are involved in transforming signals of energy availability to signals modulating GnRH secretion? Many of the potential candidate neurons express steroid receptors. Do growth-related signals about energy metabolism that regulate steroid-independent GnRH secretion also modulate sensitivity to steroid feedback inhibition of GnRH secretion? How are these internal cues integrated with external cues that provide information about the environment to the developing individual?

An important unanswered question that relates to the timing of puberty in sheep and other species is whether there exists a neural component that functions as a developmental clock (Figure 31.29)[320] i.e., is there a minimum age where puberty is genetically programmed to begin, which is then modified by internal and external cues? If so, is the innate developmental "clock" a single timer or a series of timers under species-specific genetic control, and what and where is the clock? Ojeda and his colleagues have considered how a hierarchy of gene networks might control puberty.[321] They propose a system of at least three layers of networked inputs, one tier of genes required for cell-to-cell communication, a second for genes that regulate the intercellular interactions and a third tier of higher echelon genes that transcriptionally regulate subordinate genes in the first two tiers. Further, the organization of such a hierarchy might involve the time-dependent expression of genes that would interact with pathways of extrinsic information to modify the activity of GnRH neurons to time puberty. Once these components are identified one could determine how and where this intrinsic clock-type information is integrated with extrinsic/permissive information about energy and physical environments. Does such a putative pubertal clock in sheep strike early in development, thereby signaling for reduced sensitivity to steroid feedback, only to await the integration of permissive extrinsic and intrinsic signals which allow for increased GnRH and LH secretion, or is timing of the pubertal clock altered in some way by extrinsic or intrinsic signals? If an integrator is involved, is it the same as that for the adult, which also uses information about

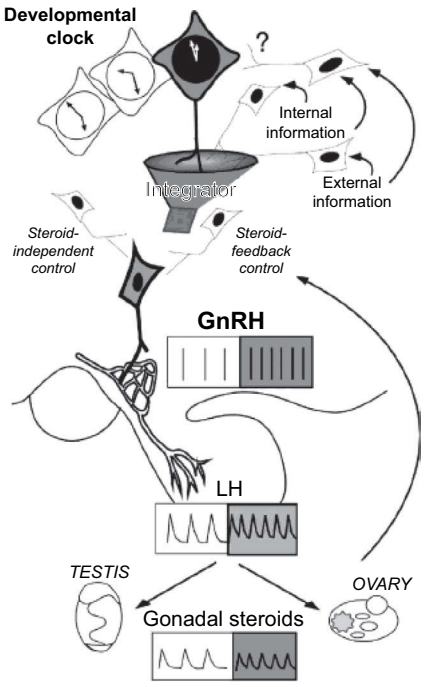

FIGURE 31.29 Model for integration of signals and control mechanisms timing puberty through the regulation of GnRH secretion. A genetically linked developmental clock is proposed to signal when it is first possible to produce the pubertal increase in GnRH secretion but the actual time when it occurs is fine-tuned by multiple permissive signals that provide information about internal and external environments. These signals regulate steroid-dependent and steroid-independent feedback control of GnRH pulse frequency. *From Ref. 287, with permission.*

energetic, social, geophysical, and other cues to time the periodic seasonal resumption of high level GnRH secretion? Armed with such concepts, and other models for a developmental clock, we can then focus on identification of its neuroanatomical location(s) and output pathways to the GnRH neural regulatory system. In going forth conceptually, the challenge will be to differentiate unique signals, sensors, and pathways from permissive ones, and from those that are used after puberty as well. Only then will we have a more complete neurobiological explanation of how reproductive activity begins for the first time.

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CHAPTER

32

Puberty in Non-human Primates and Man

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INTRODUCTION

Puberty as defined by Webster "the period of becoming first capable of reproducing sexually, marked by maturation of the genital organs, development of secondary sex characteristics, and, in the human and in higher primates, by the first occurrence of menstruation in the female",[1] requires little clarification. Of keynote importance in Webster's definition is the phrase "the period of becoming", since it correctly implies that puberty is a temporal process involving transition and development. Development may be viewed as the summation of differentiation and growth, and in highly evolved primates (Old World monkeys, apes, and humans),[a] normal puberty is observed during the terminal phase of growth. The transition into primate puberty is driven to a variable, species-dependent extent by two physiologic processes: gonadarche and adrenarche.[3] Gonadarche comprises growth and maturation of the gonads, and is associated with increased secretion of sex steroids and with the initiation of folliculogenesis and ovulation in the female and spermatogenesis in the male. Adrenarche comprises maturation of the adrenal cortex and is associated with increased secretion of adrenal androgens. While gonadarche is obviously critical for puberty in all species, adrenarche late in prepubertal development appears to be peculiar to man and the great apes.[3–7] In man, the physical manifestations of adrenarche are known as pubarche, and include the appearance of pubic and axillary hair, acne, and adult apocrine odor. The absence of adrenarche in humans does not prevent fertility, nor does it dramatically influence the timing and tempo of gonadarche,[3,8,9] and thus from a biological perspective adrenarche can be viewed as a temporal corollary of the pubertal process rather than as an integral and fundamental component of this important developmental event.

[a] The order primates comprises five major groups; Lemuriforms (lemurs), Lorisoforms (lorises and bushbabies), Tarsiiformes (tarsiers), Platyrrhini (New World monkeys and marmosets), and Catarrhini (Old World monkeys, apes, and humans)[2]. The latter infraorder is divided into two super families: Cercopithecidae (Old World monkeys) and Hominoidea (apes and humans).

[b] The following trivial names have been employed: African green monkey, *Cercopithecus aethiops*; baboon, *Papio*; bonnet monkey, *Macaca radiata*; bonobo, *Pan paniscus*; capuchin monkey, *Cebus apelle*; chimpanzee, *Pan troglodytes*; common marmoset, *Callithrix jacchus*; cotton-top tamarin, *Sanguinous oedipus oedipus*; crab-eating or cynomolgus monkey, *Macaca fascicularis*; gorilla, *Gorilla*; hanuman langur, *Presbytis entellus*; mandrill, *Mandrillus sphinx*; owl monkey, *Aotus trivirgatus griseimembra*; pigtail monkey, *Macaca nemestrina*; rhesus monkey, *Macaca mulatta*; saddle-back tamarin, *Saquinus fuscicollis*; sooty mangabey, *Cercocebus atys*; squirrel monkey, *Saimiri sciureus*; and talapoin monkey, *Miopithecus talapoin*.

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Perhaps the most striking feature of sexual development in primates is the pronounced interval that exists from birth until the initiation of the pubertal process. For example, although the sexually mature common marmoset[b] has a body weight similar to that of an adult rat (300 g), puberty in this small New World monkey is not observed until at least 6–12 months later than that in the laboratory rodent.[10] The delay in the onset of puberty is most conspicuous in our own species, where initiation of spermatogenesis and ovarian cyclicity does not normally occur until the second decade of life. Thus, puberty in primates is separated in a most definite fashion from perinatal development. In these species, therefore, identification of the initiation of the pubertal process and its physiological and behavioral concomitants is a relatively simple task. At the other end of the spectrum of mammalian development are the rapidly maturing species, such as laboratory rodents, in which the processes of neonatal differentiation and puberty are much more difficult to separate.

In man, the prolonged period of development from birth to adulthood may be divided into five life history stages, namely infancy, childhood, juvenile, adolescence (pubertal), and adulthood.[11] Infancy is defined as the period when nourishment of the offspring is obtained via suckling or from formula and ends at the time of weaning. Childhood is the period during which the offspring remain dependent on their parents (or surrogate elders) for food and protection. In contrast to children, juveniles are no longer dependent on parents (elders) for survival. The juvenile phase of development is terminated by the onset of puberty, and at this time the individual enters adolescence. Since childhood is observed only in hominids,[11] for the purpose of the present chapter the term juvenile will be used to describe the phase of development between infancy and puberty in both human and non-human primates.

In primates, as in other mammalian species, the state of having entered the development stage defined as puberty is recognized by the cascade of morphological, physiological, and behavioral sequelae of increased gonadal and, in some cases, adrenal activity at this time. In Old World monkeys, apes, and man this stage of development may span a time frame of several years. Some of the biological changes associated with puberty are relatively discrete and, therefore, provide quantitative markers of this transitional phase in development. Many others, however, are less obvious and more difficult to monitor. It has thus not been possible to pinpoint the beginning or the ending of puberty in primates with any degree of precision, nor has it been possible to provide for any of these species a complete chronicle of events that unfold as an individual progresses through puberty. It is important to appreciate at the outset that the foregoing paucity of descriptive information has minimal impact on our ability to construct and to test physiological models that have been, or may in the future be, proposed to account for the timing of puberty in primates. The purpose of this chapter is to examine the control systems that underlie the transition into puberty, rather than providing a comprehensive review of the morphological, physiological, and behavioral concomitants associated with puberty (see Ref. 3 for man). Emphasis will be placed on the highly evolved primates, which include the Old World monkeys and apes, including our own species.

The conceptual framework, which serves as the basis for our classical understanding of puberty, has been elegantly reviewed by Donovan and Van der Werff ten Bosch.[12] Only the major steps in the progression of thinking on this subject need to be reiterated here. That a major component of puberty involves a change in gonadal activity became apparent before the time of Christ, as knowledge of the effects of castration during prepubertal development in our own species first began to accumulate.[13] Similarly, the idea that the pubertal change in gonadal activity is of a humoral nature is not of modern origin and, as pointed out by Donovan,[14] dates back to at least the seventeenth century. The signal finding of Foa at the turn of the present century, that transplantation of ovaries from an immature animal into an adult host led to a premature onset of pubertal activity in the transplanted gonad,[15] provided the foundation for the view that although maturational processes may occur within the gonad of the immature animal, these are terminated long before the onset of puberty. Confirmation of Foa's original finding by several other groups led Lipschutz and his colleagues[16] to formulate in 1929 the "law of puberty", which stated that the onset of pubertal changes in the gonad is determined by maturation of a somatic component rather than by that of the gonad itself.

That the somatic component of the control system governing puberty might be the pituitary gland was suggested by the early finding of Crowe et al.[17] that partial ablation of the adenohypophysis in the prepubertal canine prevented the onset of sexual maturation. Definitive proof for the pituitary's role in puberty was provided 17 years later by Smith and Engel,[18] who convincingly demonstrated that implantation of pituitary fragments in immature rats and mice led to premature sexual maturation in these species. However, with the subsequent forging of contemporary understanding of the neural regulation of anterior pituitary function by Geoffrey Harris and others,[19] the role of the pituitary gland in puberty was relegated to that of a slave to the brain. In a fashion analogous with Foa's earlier ovarian transplantation experiments, Harris and Jacobsohn[20] found that when pituitary glands taken from immature rats were transplanted to a site beneath the median eminence of their hypophysectomized mothers, estrous cyclicity was reinitiated in the recipient animals well before puberty would have been

expected to occur in the donors if the latter had remained intact. This result provides compelling evidence in support of our current concept that an intrinsic immaturity of the pituitary is not the cause of the relative ovarian and testicular quiescence during prepubertal development in many mammalian species, including primates.[21]

The finding from one of the authors' laboratories that chronic intermittent neurochemical stimulation of the hypothalamus of the prepubertal monkey imposes a precocious adult pattern of activity in the hypothalamus–pituitary–testicular axis indicates that the network of gonadotropin-releasing hormone (GnRH)[c] neurons, which provide the major drive to this axis, must also be viewed as a nonlimiting component of the control system that governs the timing of puberty.[22] Instead, the onset of puberty appears to depend on the ontogeny of central neural mechanisms that are upstream of the GnRH neuron and are able to dictate the postnatal activity of these cells. The critical importance of the central nervous system (CNS) in the pubertal process in primates is graphically reinforced by an extensive clinical literature documenting an association between lesions of the human brain, particularly those of the hypothalamus and adjacent areas, and disorders of puberty, such as GnRH-dependent precocious and delayed gonadarche.[3] With the discovery in 2003 that loss of function mutations in G-protein coupled receptor 54 (GPR-54; also known as kisspeptin receptor, KISS1R) is associated with hypogonadotropic hypogonadism and delayed or absent puberty in man,[23,24] the idea that hypothalamic kisspeptin signaling is the single most important upstream regulator of the GnRH neuronal network has emerged.

GONADAL AND ADRENAL FUNCTION DURING PUBERTY

The Testis

The growth of the human testis during the first decade of postnatal life is unremarkable. A dramatic increase in testicular volume between 9 and 13 years from 2 ml to the adult volume ranging from 20 to 25 ml[3] marks male gonadarche. The chimpanzee exhibits a pattern of testicular growth similar to that of humans, although the phase of pubertal enlargement is initiated at approximately 6 years of age.[25–28] In rhesus, crab-eating, and bonnet monkeys and in baboons, there is also little growth of the testis during prepubertal development.[29–33] At around 3 years of age in these species of Old World monkey, testicular size increases markedly within a period of 1.5 years, from a volume of approximately 2.5 ml to an adult capacity of 25 ml. In the mandrill, pubertal testicular enlargement is observed between 4 and 8 years of age.[34] A similar developmental pattern of testicular growth has been reported for the common marmoset, although, in the case of this New World primate, initiation and completion of the pubertal phase of rapid growth take place at a much earlier age than in highly evolved primates.[10] In two other species of platyrrhine, the owl monkey and the capuchin monkey, a marked pubertal acceleration in testicular growth was only noted in the latter species.[35,36]

The testes of postinfantile, prepubertal macaques and humans contain few, if any, recognizable Leydig cells,[37–40] and testicular testosterone secretion during this phase of development, in these and other species of primate, is minimal.[40–52] The precise temporal relationship among the acceleration in testicular growth, the development of Leydig cells, and the increase in testosterone secretion during early puberty has not been systematically studied in any species. The development of Leydig cells and a rise in intratesticular androgen concentration, however, likely precede both the acceleration in testicular growth and the rise in circulating androgen levels. In humans, daytime levels of plasma testosterone begin to rise above prepubertal concentrations of 0.2 ng/ml during the tenth year of life and continue to increase progressively before reaching, at 14–15 years of age, a value (6 ng/ml) typical of that observed in adult men.[41,42,53] The initiation of the pubertal rise in circulating daytime testosterone levels has been observed to occur between the ages of 6 and 8 years in the chimpanzee[28,43]; 4 and 8 years in the mandrill[34]; 2.5 and 4 years in the rhesus, crab-eating, and African green monkeys, the sooty mangabey, and the baboon[45,47–52]; 2.5 and 3 years in the squirrel monkey[46]; and approximately 1 year in the common marmoset and the owl monkey.[10,35] Studies of humans and the rhesus monkey show that initial activation of testicular testosterone secretion during early puberty occurs nocturnally.[49,54–56] In the rhesus macaque, a nighttime increment in circulating testosterone concentrations may be observed as early as 2–3 years of age, several months before the onset of the pubertal rise in daytime concentrations of this steroid.[49] Although longitudinal studies of the peripubertal time course in testosterone secretion are scant, the available data for humans[57,58] and the rhesus monkey[33] suggest that the transition from a prepubertal to an adult pattern of secretion may occur relatively briskly. Longitudinal studies of sexual development in humans also reveal a striking increase in testicular growth during puberty.[12]

The age at which spermatogenesis is initiated has been less precisely defined than that for the pubertal onset of steroidogenic activity by the Leydig cell because of the difficulty in obtaining serial ejaculates

[c] Throughout the chapter we will use GnRH to describe GnRH-1 (mammalian GnRH).

and testicular biopsies. Limited data are available for macaques and humans only, and no attempt has been made to systematically relate the maturation of seminiferous tubule morphology to the pubertal initiation of Leydig cell function. In contrast to the steroidogenic component of the primate testis, which appears to be quiescent during the postinfantile, prepubertal stage of development, the seminiferous cords lengthen during this phase of development due to proliferation of Sertoli cells and undifferentiated type A spermatogonia.[33,37,38,59–62] The acceleration in testicular growth during early puberty results largely from an increase in diameter and tortuosity of the seminiferous tubules associated with the appearance of mature Sertoli cells and proliferation of germ cells. Although spermatogenesis has been generally recognized to become established between the 12th and 16th year of life in humans,[37,59] studies of the incidence of spermaturia in boys suggest that the onset of sperm production may occur in association with the initiation of accelerated testicular growth.[63,64] In a qualitative sense, the postnatal ontogeny of the germinal epithelium in macaques appears comparable to that described for humans. In these Old World monkeys, sperm are first observed in the seminiferous tubules between 3 and 4 years of age.[32,33,38,60] In New World monkeys, spermatogenesis is probably initiated during the first year of life; spermatocytes have been reported in the marmoset testis at 35 weeks of age,[10] and cotton-top tamarin males as young as 22 months of age may impregnate female.[65]

The Ovary

Knowledge of the ontogeny of the primate ovary has been derived primarily from studies of the human and rhesus monkey.[66–70] In contrast to the postnatal pattern of testicular growth, the size of the ovary increases in a rectilinear fashion from infancy to adulthood. Moreover, growth and atresia of ovarian follicles begin in utero and continue during the prepubertal years. From a peak of approximately six to seven million oocytes in the human female fetus at midgestation, some 350,000 remain at birth due to losses secondary to apoptosis.[71,72] Enlargement of the ovary prior to puberty is the result of age-related increases in the number and size of antral follicles and in the quantity of medullary stroma independent of gonadotropin stimulation. Sonography shows small follicles in the ovaries from the majority of prepubertal girls with increasing variation in follicular size postmenarche.[3] Although the size attained by a growing antral follicle before atresia intercedes is variable, Graafian (preovulatory) follicles are not observed during prepubertal development. Whereas primordial and pre-antral follicles predominate during the prepubertal years, small antral follicles can develop prior to puberty. With gonadotropin stimulation at the initiation of puberty, the adolescent ovary may appear on ultrasound images to be multi-cystic.[73]

The availability of sensitive and specific methodology to measure the low serum estradiol concentrations in prepubertal girls and non-human primate females is limited. Two observations in prepubertal female monkeys, however, demonstrate that the prepubertal ovary actively secretes estradiol. First, ovarian vein concentrations of estradiol are three- to fourfold greater than peripheral levels,[74] and second, circulating estradiol concentrations decline following ovariectomy.[75] That the prepubertal human ovary also secretes estradiol is supported by the finding, obtained with an ultrasensitive estradiol bioassay, that circulating levels of this steroid are significantly greater in prepubertal girls than in boys.[76,77] Cross-sectional studies of girls have demonstrated that between 8 and 10 years of age, estradiol concentrations in serum samples collected during the morning begin to increase to values typically seen in adult women during the early follicular phase of the menstrual cycle.[78–80] Concomitant with the rising estradiol concentrations, the early clinical feature of gonadarche, namely the initiation of breast development, occurs. Uterine volume increases throughout puberty, with the greatest increase occurring during midpuberty.[81] In the female rhesus monkey, circulating estradiol concentrations rise between 2.5 and 3 years of age in association with nipple growth and an increase in perineal swelling and coloration.[82,83]

In the human female, further development of secondary sexual characteristics occurs as puberty progresses and culminates in menarche, which generally occurs 2–3 years following the onset of breast development.[3] The Third National Health and Nutrition Examination Survey (NHANES III), conducted between 1988 and 1994, reported the average age at menarche to be 12.54 years in North American girls, with an average age estimated to be 12.6 years for whites and 12.14 years for blacks.[84] Menarche presumably represents acute withdrawal of hormonal support of estrogen-induced endometrial proliferation. Typically, menstrual cycles during the first year after menarche are often irregular and anovulatory,[85–90] with most ranging in duration from 21 to 45 days. Within 5 years of menarche, the majority of cycles are regular and range from 21 to 35 days. Fertility during the peripubertal years appears to be compromised by a relatively high incidence of menstrual cycles with short luteal phases.[87,89,90]

Although menarche occurs in the great apes and Old World monkeys at a younger age than that in the human female (Table 32.1), the postmenarcheal phase of development in these species is also characterized by a variable phase of adolescent infertility[85,91,94,97] that, in the case of the rhesus monkey, is the result of a high incidence of anovulatory and short-luteal-phase cycles.[103,104]

TABLE 32.1 Age at Menarche in Old World Monkeys and Great Apes

Species	Age (year)	Reference
Chimpanzee	7–11	91–93
Gorilla	6–8	94
Baboon	3–4	95,96
Talapoin monkey	3–4	97
Pigtail monkey	2–4	98
Rhesus monkey	2–3	99,100
Crab-eating monkey	2–3	101
Hanuman langur	2–3	102

In the common marmoset and squirrel monkey, species of New World primate in which menstruation is not observed, ovulation as indicated by elevated plasma progesterone levels and conception may occur as early as 2.5–3 years of age.[10,46] In other species of platyrrhine monkey—for example, the saddle-back tamarin, in which conception has been reported as young as 210–230 days of age[105]—the transition into puberty occurs within the first year of life under appropriate environmental and social conditions (see below).

The Adrenal

The adrenal cortex consists of three histologically and functionally distinct zones; the zona glomerulosa produces mineralocorticoids (aldosterone), the zona fasciculata produces glucocorticoids (cortisol), and the reticularis produces the C-19 adrenal androgens, dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEAS), and androstenedione. The pituitary regulates circulating cortisol concentrations through secretion of adrenocorticotropin (ACTH), a peptide derived following proteolytic cleavages of proopiomelanocortin (POMC). In the adrenal cortex, ACTH binds to its cognate receptor, a G-protein coupled 7-transmembrane domain receptor that signals via cyclic AMP and protein kinase A. ACTH has both acute and chronic effects on the adrenal cortex. Acutely, ACTH promotes uptake of low density lipoprotein, stimulates cholesterol esterase activity, enhances synthesis and phosphorylation of steroid acute regulatory protein, and promotes cortisol secretion. The chronic effects of ACTH involve stimulation of transcription and translation of steroidogenic enzyme genes. ACTH secretion is controlled by hypothalamic releasing factors, such as corticotropin-releasing hormone (CRH) and vasopressin.

Adrenarche refers to the increased secretion of C-19 steroids (DHEA, DHEAS, and androstenedione) by the zona reticularis that begins at 6–7 years of age in humans.[3] Circulating DHEAS concentrations peak during the second decade of human life and, then, progressively decline.[106] While designated as adrenal androgens, these steroid hormones do not activate the androgen receptor. Rather, they serve as precursors for testosterone and dihydrotestosterone in target tissues.

In contrast to the role of hypothalamic GnRH in the initiation of gonadarche (see below), CRH and vasopressin do not appear to trigger the onset of adrenarche. Adrenarche occurs independently of developmental changes in the hypothalamic–pituitary–gonadal axis. For example, children with gonadal dysgenesis experience normal adrenarche and pubarche (growth of pubic hair), whereas children with primary adrenal insufficiency may have normal gonadarche.[3,8,107] No significant changes in ACTH concentrations have been noted during adrenarche, and no specific adrenal androgen-stimulating factors have been isolated from the pituitary. Nevertheless, decreased DHEAS concentrations in patients with ACTH resistance associated with mutations in the ACTH receptor gene (*MC2R*), and patients with ACTH deficiency, indicate that ACTH plays a permissive role in adrenarche.[108] Patients with ACTH resistance fail to experience adrenarche and females have poor pubic hair development.[108] Available data suggest that undefined gradual developmental changes in the enzymatic machinery responsible for steroidogenesis within the adrenal cortex underlie adrenarche.[3]

PUBERTAL STAGING

The physical changes associated with the onset and progression of puberty in highly evolved primates have been systematically defined only in man. Specifically, the genital and pubic hair changes that emerge in association with the onset of puberty in boys and girls are classified into five stages: Tanner stage 1 is prepubertal and Tanner stage 5 is adult.[3] These physical changes may be the result of either gonadarche, as in the case of breast or testicular enlargement, or adrenarche, as in the case of pubic hair development, particularly in girls. Although the physical sequelae of gonadarche and adrenarche generally occur concomitantly, a discordance of the two processes may also be observed in normal development.

Increased estrogen secretion promotes breast development in girls. The development of breast buds with increased areolar diameter is considered to be stage 2, and greater enlargement of the breasts occurs in stage 3 accompanied by increased pigmentation of the areolae and nipples. During stage 4, the areolae are mounded above the breast tissue. Recession of the areola to the general breast contour represents breast stage 5. Additional effects of estrogen at this stage of development include cornification of the vaginal mucosa, uterine growth, and morphogenesis of an adult female body habitus.

In girls, increased adrenal androgen secretion is considered to be responsible for the development of darker hairs usually along the labia, which is classified as pubic hair stage 2. During pubic hair stage 3, the hair is darker and coarser, spreading over the pubic symphysis with gradual progression to a full female escutcheon. Apocrine odor may precede or accompany the development of pubic hair. Associated findings include acne and oiliness of skin and hair.

For boys, an increase in testicular volume is considered genital stage 2. At stage 2, the testes are approximately 4–8 ml in volume with the longest axis being approximately 2.5 cm. With increasing testicular volume, the scrotum enlarges and is accompanied by decreased rugation of the skin. The volume of the mature human testis is approximately 20–25 ml and represents increased growth of the seminiferous tubule due to Sertoli cell proliferation and differentiation, and initiation of spermatogenesis. At genital stage 3, further growth of the testes has occurred, and the length and diameter of the penis has increased. At genital stage 4, penile size has increased with darkening of the scrotal skin.

Male pubic hair stage 2 consists of downy hair at the base of the penis. For pubic hair stage 3, the hair is longer and darker, and extends over the junction of the pubic bones. For pubic hair stage 4, the extent of hair has increased, but has not yet achieved the adult male escutcheon. Other secondary sexual characteristics in boys include increased size of the larynx, deepening of the voice, increased bone mass, and increased muscle strength. Approximately 3 years after the appearance of pubic hair, terminal hair appears in androgen-dependent regions on the face and trunk, where it may develop for years thereafter. The distribution and density of beard, chest, abdominal, and back hair vary considerably, presumably reflecting genetic differences. The appearance of spermatozoa in early morning urine specimens (spermaturia) occurs during genital stage 3. Transient gynecomastia (breast development) is observed in approximately 50% of boys.[109] Typically, this is most prominent in midpuberty when the ratio of circulating concentrations of estradiol to testosterone is relatively high.

The pubertal growth spurt in girls occurs concurrently with the onset of breast development. Usually only 4–6 cm of growth occur after menarche. In boys, the pubertal growth spurt, with an average height velocity of 9.5 cm/year, occurs around genital stages 3–4. In general, the age at peak height velocity shows an inverse relationship with the magnitude of the growth spurt.

A timeline of pubertal staging in female rhesus monkeys based on measurements of physical parameters and reproductive hormones has been established.[82] The first sign of female puberty in this species is a slight increase in the nipple volume, which is followed by an increase in perineal sex-skin swelling. During this period acceleration of body weight also occurs. These somatic changes are due to a small increase in circulating estradiol levels. Menarche occurs at around 30 months of age. Increases in nipple volume, sex-skin swelling, estradiol levels and body weight are further accelerated until the time of first ovulation around 42 months of age, after which all of these parameters reach a plateau. Thus, the pubertal stages in female rhesus monkeys are defined as follows: "prepubertal": no sign of puberty; "early pubertal": the period between the first sign of puberty and menarche; and "midpubertal": the period between menarche and first ovulation. These stages correspond to Tanner stages 1, 2, and 3, respectively, in girls.

SECULAR TRENDS

Over the last century and a half, the age of menarche in North America, Europe, and other industrialized areas of the world appears to have decreased dramatically from 16 to 17 years of age in the middle of the nineteenth century to 12–13 years of age in the late twentieth century.[3,9,110–113] This well-established trend toward an earlier age at menarche, which in the early twenty-first century may be coming to a halt,[3,114–119] is generally attributed to a corresponding secular improvement in the standard of living, including better nutrition and health care. Additionally, environmental contamination, with agents such as bisphenol A, is also considered to be a possible cause of early puberty.[120,121] Although analogous data for puberty in the human male are scant, in a recent study of American boys who were taller and heavier than those in previous cohorts, pubic hair and testicular development were reported to occur earlier than previously recognized.[122] Interestingly, a secular trend toward an earlier age of menarche has also been reported for the rhesus monkey in laboratory settings, and this is attributed to an increased calorie intake.[99,123]

PITUITARY GONADOTROPE FUNCTION DURING PUBERTY

Conceptually, it is useful to define two modes of gonadotropin secretion, namely basal (or tonic) and surge. The tonic mode accounts for the secretion of these hormones in the male, and for the relatively low circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels observed in females during the greater part of the menstrual cycle (see Chapter 28 for details). In the male, this mode of secretion is responsible for maintaining spermatogenesis and stimulating testosterone secretion. In the female, tonic gonadotropin secretion drives folliculogenesis and estradiol production in the follicular phase and maintains the corpus

luteum in the second half of the cycle. The surge mode of gonadotropin secretion, which results in the massive mid-cycle discharge of LH and FSH, triggers ovulation, and is termed the preovulatory gonadotropin surge.

Our understanding of tonic gonadotropin secretion during puberty in primates has been obtained primarily from studies of humans, and these were reviewed in detail in the last edition of this book.[124] With the advent, in the late 1960s, of radioimmunoassay (RIA) for the determination of LH and FSH in small volumes of serum, a large number of research groups examined, in comprehensive cross-sectional studies, the time courses of circulating LH and FSH concentrations in normal boys and girls during pubertal development. The general statement may be made that the transition into puberty in both boys and girls is tightly coupled with an initiation of a progressive increase in the overall secretory activity of the pituitary gonadotropes, as reflected by the time courses in circulating LH and FSH concentrations during this phase of human development. With the advent of the more sensitive immunoradiometric or immunofluorometric assays for the measurement of blood gonadotropin concentrations, it is now clear that FSH and LH levels rise concomitantly at puberty in both boys and girls, and that the increase in LH at this stage of development is particularly robust. In the case of LH, where bioassay has also been applied, the pubertal rise in this gonadotropin is revealed to be an immediate, rapid, and striking event.

Although studies of the time courses of basal gonadotropin secretion during juvenile and pubertal development in non-human primates, specifically, the chimpanzee,[28,125] the macaques,[30,31,49,52,83,126] and the sooty mangabey,[48] have been collectively less comprehensive than those of humans, the coupling between a rise in circulating LH and FSH concentrations and the transition into puberty is a general characteristic of sexual maturation in highly evolved primates. Indeed, the switch to an LH-dominated pattern of gonadotropin secretion at puberty is reflected in the male rhesus monkey by a greater proportion of LH immunoactive gonadotrophs in the pituitary of the adult compared with that of the juvenile.[127]

In boys and the male rhesus monkey, the pubertal increase in gonadotropin secretion is characterized by nocturnal elevations of LH concentrations that drive nighttime testosterone secretion characteristic of this stage of development.[49,54,55,128–133] Increased nocturnal activity in the pituitary–Leydig cell axis is sleep related in boys.[134] The pubertal activation of the pituitary gonadotrope in the female is also characterized by nocturnal elevations in LH secretion,[124,135–138] which, in the case of the human female, are also sleep related.[135,136]

It is also during the pubertal phase of development that spontaneous preovulatory gonadotropin surges are first observed in the female[d]. In the rhesus monkey, and probably in humans, this takes place after menarche.[82,139–141]

The fundamental role of increased gonadotropin secretion to initiate gonadal development at puberty is well established. In humans, spermatogenesis and ovulation may occur at very young ages as a result of premature gonadotropic stimulation of the gonad.[3,142–144] Similarly, stimulation of the gonad in the prepubertal rhesus monkey with either exogenous or endogenous gonadotropin leads to the premature onset of ovulation and spermatogenesis.[21,22,145–148] Also, chronic stimulation of gonadotropin secretion in subjects with delayed puberty secondary to hypogonadotropic hypogonadism results in the pubertal-like activation of the testis and ovary and fertility.[149–152] Conversely, a delay in the onset of puberty in humans is associated with low circulating gonadotropin concentrations and hypogonadism,[3,9,153] and inhibition of gonadotropin secretion in individuals with precocious gonadarche results in a return to testicular and ovarian quiescence.[3,143,144,154]

Taking the foregoing findings together it is clear that an understanding of the physiological mechanisms that regulate the timing of puberty requires an appreciation of the control system that governs gonadotropin secretion and its development.

NEUROENDOCRINE CONTROL OF GONADOTROPIN SECRETION IN THE ADULT

Before embarking on a discussion of the development of the neuroendocrine control system that governs gonadotropin secretion, we provide perspective by presenting a brief overview of the essential features of the regulation of gonadotropin release in the adult. For detailed examinations of this subject in the female, the reader is referred to Chapter 28. The primary stimulus to pituitary gonadotropin secretion is generated within the CNS and is transmitted to the gonadotropes of the adenohypophysis via the hypophysial portal circulation by an intermittent hormonal signal in the form of the hypothalamic releasing factor, GnRH. Parenthetically, it should be noted that a second GnRH (GnRH-II) is also found in the primate hypothalamus,[155,156] and the gene encoding the GnRH-II receptor is expressed in the anterior pituitary,[157,158] although the human gene encoding for this receptor does not generate a functional protein.[159,160] Although intravenous administration of GnRH-II to the rhesus monkey is able to elicit LH release,[161] and in this species GnRH-II is developmentally regulated[156] and estrogen responsive,[162] the role of

[d] It is to be noted that the ability of estradiol to elicit gonadotropin surges during infantile development has not been tested.

this peptide in regulating the primate pituitary–gonadal axis is unclear. Similarly, an RF amide-related peptide, RFRP3, that is considered to be a homolog of gonadotropin-inhibitory hormone has been identified in the mammalian hypothalamus,[163] but its neuroendocrine role in primates has yet to be established.

The distribution of GnRH perikarya and their projections within the primate hypothalamus has received considerable attention (see Chapter 11), and insight into the neurobiology of the hypothalamic GnRH pulse generator is beginning to emerge. In the primate hypothalamus there are some 1000 to 2000 diffusely distributed GnRH neurons, many of which send their projections to the median eminence where they synchronously and intermittently discharge their peptide into the primary plexus of the hypophysial portal circulation, thereby providing the pituitary gonadotrophs with the pulsatile stimulation that, as first demonstrated by Knobil, is essential for maintaining gonadotropin secretion (see Chapter 28). Recently, these GnRH projections to the median eminence in mice were recognized to share characteristics of axons and dendrites and were accordingly termed dendrons[164]: it is speculated that communication among multiple GnRH neurons underlying synchronous peptide release may occur at the dendrons (see Chapter 11). While the neurobiological mechanisms that underlie GnRH pulse generation remain controversial, compelling evidence is emerging to indicate a fundamental role of KNDy neurons in the arcuate nucleus (also called infundibular nucleus).[165–168] These hypothalamic neurons, named because they coexpress *k*isspeptin, *n*eurokinin B (NKB) and *d*ynorphin,[169] project their axons to the median eminence where they mingle intimately with GnRH fibers en route to the portal vessels.[170] It is proposed that GnRH pulse generation is achieved by reciprocating stimulatory (NKB) and inhibitory (dynorphin) connections within the arcuate nucleus, while the output of the pulse generator is relayed to GnRH fibers projecting to the median eminence by an intermittent kisspeptin signal. Thus, these neurons may represent the anatomic site of the GnRH pulse generator. Loss of function mutations in the genes encoding for kisspeptin (*KISS1*) or *KISS1R* and for NKB (*TAC3*) or the NKB receptor (*TACR3*) in man lead to a profound hypogonadotropic state that is typically manifest by the absence of gonadal development at the expected age of puberty.[23,24,171,172] On the other hand, gain of function mutations of both *KISS1* and *KISS1R* has been implicated in the etiology of precocious pubertal GnRH-dependent gonadotropin secretion in man.[173,174] The mechanism of action of the receptor mutation has been attributed to delayed degradation of the KISS1R leading to a prolonged duration of action.[175] The importance of KNDy neurons has also been demonstrated by the finding that their ablation either nonselectively in the adult monkey or selectively in the postpubertal rat results in a loss in hypothalamic drive to LH secretion.[176,177] Contemporary models for GnRH pulse generation are discussed further in Chapters 27 and 28.

The hypothalamic GnRH pulse generator and the pituitary gonadotropes comprise the basic neuroendocrine unit responsible for basal/tonic LH and FSH secretion. Tonic gonadotropin release in the adult is regulated by negative feedback actions of the gonadal hormones on LH and FSH secretion that may be exerted either directly on the gonadotropes to suppress their responsivity to GnRH stimulation or indirectly at a suprapituitary locus to modulate the frequency and/or amplitude of pulsatile GnRH release. Removal of such negative feedback influences results in a chronic hypersecretion of both LH and FSH, which occasions a sustained elevation in circulating gonadotropin concentrations an order of magnitude higher than those observed in intact subjects. In the absence of feedback by gonadal hormones, the hypothalamic GnRH pulse generator of sexually mature male and female primates appear to exhibit an operational frequency of approximately one cycle per hour. This "adult" pattern of intermittent GnRH release, as measured directly in the monkey or reflected in the circhoral discharge of pituitary LH release, has been observed in male and female rhesus monkeys castrated postpubertally, in ovariectomized and post-menopausal women, and in men with primary gonadal failure or following castration (see Chapter 28).

The primary testicular components of the negative feedback loops governing gonadotropin secretion in the male are testosterone and inhibin B, which regulate LH and FSH, respectively. In the female, tonic gonadotropin secretion, which is observed during the greater part of the follicular phase and during the luteal phase of the menstrual cycle, is controlled by a negative feedback action of ovarian hormones (primarily estradiol) on LH and FSH release.

In addition to tonic gonadotropin secretion that drives folliculogenesis and maintains the corpus luteum, the female also requires expression of the surge mode of gonadotropin secretion to achieve ovulation. This mode of gonadotropin secretion is generated by a stimulatory or positive feedback action of estradiol on LH and FSH release. The positive feedback action of estradiol is elicited, at least in the human and macaque female, when circulating levels of this steroid secreted by the ovarian follicle destined to ovulate are maintained for approximately 36–48 h at a threshold concentration of 200–500 pg/ml during the late follicular phase of the cycle. The precise neuroendocrine mechanism that is activated by the positive feedback action of estradiol in highly evolved primates and that therefore underlies the preovulatory gonadotropin surge in these species is poorly understood. In this regard, the concept of

distinct anatomical centers in the medial basal hypothalamus (MBH) and rostral hypothalamus that govern tonic gonadotropin secretion and the preovulatory discharge, respectively, which was originally proposed to account for LH secretion throughout the estrous cycle of the rat, is generally omitted from current models of the primate menstrual cycle.[178] Whether the hypothalamic machinery responsible for the preovulatory gonadotropin surge in primates involves more than the GnRH pulse generator and the GnRH neurons that it regulates is unclear. However, there is considerable evidence from GnRH deficient human and monkey models to support the view that pulsatile GnRH stimulation, alone, is sufficient for the generation of the preovulatory gonadotropin surge. Moreover, the postnatal development of surge gonadotropin secretion has been scantly studied in primates. For the foregoing reasons, only the role of hypothalamic GnRH pulse generation in the development of tonic gonadotropin secretion and the onset of puberty will be considered in detail.

If GnRH pulse generator activity is compromised in the adult, as occurs, for example, in severe undernutrition and during strenuous exercise training, individuals become hypogonadotropic and hypogonadal (see Chapters 35 and 36). Thus, the pituitary gonadotrophs may be viewed as a slave to the hypothalamic GnRH pulse generator, and this analogy should be borne in mind as the developmental changes in gonadotropin secretion are considered.

DEVELOPMENTAL PATTERN OF GONADOTROPIN SECRETION

Fetal

Although descriptions of the circulating LH and FSH concentrations during fetal development in primates are incomplete, it has been established for humans and rhesus monkeys that secretion of these hormones is robust during mid- to late pregnancy,[9,179–181] when their levels in the fetal circulation may attain values comparable to those observed in adult castrates. The magnitude of the fetal rise in circulating FSH and LH concentrations is most striking in the female.[179–181e] An inhibitory action of fetal testicular hormones on LH and FSH secretion at the hypothalamic and/or pituitary level provides the most likely explanation for the relatively lower circulating LH and FSH concentrations in the male at this stage of development. This view is supported by the finding of Resko and Ellinwood,[182] demonstrating that orchidectomy between 98 and 104 days of fetal development in the rhesus monkey elicits a dramatic postcastration hypersecretion of gonadotropin that, within 3 weeks, results in a rise in circulating LH and FSH to concentrations observed in intact female fetuses of comparable age. Ovariectomy at this stage of fetal development does not appear to influence gonadotropin secretion.

During the latter half of human gestation, FSH and LH concentrations in the fetal circulation decline, and, at the time of birth, low gonadotropin levels are consistently observed in cord blood.[183] A similar suppression of gonadotropin secretion during late fetal development is also observed in the rhesus monkey,[181] although in the case of this species, elevated LH and FSH concentrations are sustained for a relatively greater proportion of fetal development before declining abruptly at the end of gestation.

Circumstantial evidence suggests that the principal stimulus to the fetal gonadotropes is provided, as in the case of the adult, by an intermittent pattern of GnRH discharge by the hypothalamus. In humans, the hypothalamic–hypophysial portal circulation and the major hypothalamic nuclei appear to differentiate by midgestation.[179] Interestingly, GnRH neurons, in contrast to other neurons in the CNS, originate outside the brain in the nasal placode and migrate to the hypothalamus during the first trimester of fetal development.[100] In the rhesus monkey, GnRH neurons reach the adult pattern of distribution within the hypothalamus early in the second trimester of gestation.[184,185] Immunoactive GnRH has been extracted from human fetal hypothalamus in the first trimester,[186] and at this stage of gestation the distribution of GnRH perikarya and their projections in the fetal brain of humans as revealed by immunocytochemistry appear similar to that in adults.[187,188] The gonadotropes in the fetal pituitary gland of humans and rhesus monkeys are able to respond to stimulation with synthetic GnRH.[189,190] Studies employing chronically catheterized ovine fetuses, a species in which the ontogeny of gonadotropin secretion during fetal development appears to resemble that in highly evolved primates,[191] have revealed the presence of distinct LH pulses during this phase of development.[192] Such discharges of LH are presumably occasioned, as in the adult, by the episodic release of GnRH by the hypothalamic pulse generator of the fetal brain.

The factors responsible for the characteristic bell-shaped time course in gonadotropin secretion during fetal development in humans are unclear. The best explanation for this phenomenon is that proposed by Grumbach and his colleagues.[9,179] These investigators suggest that differentiation of pituitary gonadotropes and the initiation of a hypophysiotropic drive to these cells are

[e] Although Kaplan et al.[179] did not observe a sex difference in fetal LH concentration in blood, the latter workers did observe that fetal pituitary LH content is greatest in females.

established early in fetal development and precede the ability of this neuroendocrine complex to respond to the inhibitory actions of gonadal and placental steroids on LH and FSH release. Thus, during the first half of fetal development, gonadotropin secretion is unrestrained and increases to a level reminiscent of that observed in the open-loop situation in adults. Thereafter, the fetal hypothalamic–pituitary unit presumably acquires the capacity to respond to the negative feedback actions of sex steroids, and circulating LH and FSH concentrations decline throughout the second half of fetal development as the placental production of estrogen and progesterone rises. That gonadotropin secretion in the primate fetus is restrained during late gestation by the inhibitory action of placental steroids is further suggested by the observation that, following parturition and the resulting removal of placental influences, circulating LH and FSH rise to concentrations markedly greater than those observed later in prepubertal development.[9,183,193–201]

Neonatal/Infant

Heightened gonadotropin secretion during neonatal development is observed in boys, and male chimpanzees, Old World monkeys, and marmosets, and is associated with marked elevations in testicular testosterone secretion that produce circulating levels of the steroid comparable to those observed in adults.[30,194,195,199,200,202] Continuous treatment of infantile male rhesus and marmoset monkeys with a GnRH receptor agonist results, as in adults, in a complete suppression of the pituitary–testicular axis,[203,204] and in normal infantile boys LH secretion is pulsatile[205,206]: findings indicating that gonadotropin secretion at this stage of development is driven by pulsatile hypothalamic GnRH secretion. Further study of the rhesus monkey has led to the notion that the hypothalamic component of the control system that governs testicular function is fully mature by the neonatal stage of development. Bilateral orchidectomy at 1 week of age elicits a post-castration rise in LH and FSH secretion seemingly indistinguishable from that observed after castration in sexually mature animals,[197] demonstrating the integrity of the negative feedback control of pituitary gonadotropin secretion during infantile development. Moreover, in such neonatal castrates, LH secretion is episodic and exhibits a pulse frequency typical of open-loop LH secretion in animals castrated postpubertally, namely, one pulse per hour.[207] The latter finding suggests that the hypothalamic GnRH pulse generator of the infantile male primate, like that of the postpubertal animal, is capable of generating the adult circhoral pattern of intermittent GnRH secretion. In addition, infantile male rhesus monkeys exhibit a diurnal variation in testicular testosterone secretion that is quantitatively similar to that typically observed in pubertal and fully adult animals and is correlated, as in adults, with a corresponding diurnal variation in LH release.[199,208,209] The latter observation suggests that the hypothalamic GnRH pulse generator of the infant is probably modulated by the same exteroceptive factors that regulate testicular function in the adult. Thus, in highly evolved primates the hypothalamic–pituitary unit that governs testicular function differentiates to full maturity during fetal development.

The hypothalamic–pituitary unit in female primates (humans, chimpanzees, and rhesus monkeys) also exhibits heightened activity during early neonatal development, resulting in an elevation in circulating LH and FSH concentrations, which are maintained for a variable period of time during infancy, depending on the species.[183,193,196,198,200,201,210] Although the foregoing findings support, at first sight, the view that the female hypothalamic–pituitary unit is also fully mature by birth, this question is worthy of further consideration. In infantile girls, circulating FSH concentrations are sustained at elevated levels for several years and often exceed, in dramatic fashion, those observed in adults during the follicular phase of the menstrual cycle, rising on occasion into the adult castrate range.[183,196,201] Circulating LH, on the other hand, exhibits a developmental pattern similar to that observed in the infantile boy, with a rise to low adult levels during the first few months of postnatal life.[183,193,196,200,201] This sex difference in gonadotropin secretion during infantile human development, which has been detected as early as the first day of postnatal life[211,212] and is observed in the late-gestation fetus,[180] results in an elevated ratio of FSH to LH in the peripheral circulation of the female. A similar sexual dimorphism has also been observed in the chimpanzee and the rhesus monkey.[182,198,208,213]

Studies of the rhesus monkey suggest that differential gonadal feedback signals cannot account for this sex difference in gonadotropin secretion: castration at 1 week of age does not abolish the difference in the time courses of gonadotropin secretion during the subsequent 40 weeks of life.[210] Similarly, in anorchic infantile boys, circulating FSH concentrations are lower than those in age-matched patients with Turner's syndrome.[212] Moreover, in agonadal infantile monkeys the relatively high FSH:LH ratio in the female is associated with a frequency of pulsatile LH secretion (one pulse every 4 h) that is markedly slower than that in age-matched castrated males (one pulse/h) (Figure 32.1). The latter finding, in turn, suggests that the hypothalamic GnRH pulse generator of the infantile female, in contrast to that of the neonatal male, is not capable of operating at the adult circhoral frequency. This may be of considerable conceptual importance, because if subsequent study establishes that the slow frequency of hypothalamic GnRH discharge in the infantile female reflects an "immature" pulse generator that fails to develop the capacity to operate at the adult

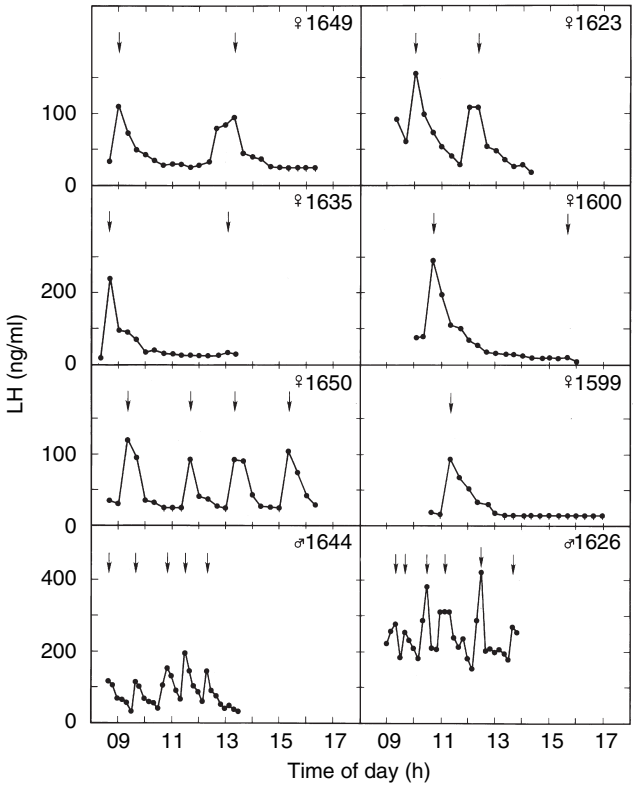

FIGURE 32.1 Moment-to-moment changes in circulating LH concentrations[f] at 4–7 weeks of age in six infantile female (top three panels) and two infantile male (lower panel) rhesus monkeys that were bilaterally gonadectomized at 1 week of age. *Arrows* indicate increments in LH identified as pulses by PULSAR. Note that the frequency of LH pulses, and presumably therefore of GnRH discharges, in agonadal infantile females is markedly slower than that in agonadal males of similar age. *Source: Reprinted with permission from Ref. 210.*

circhoral frequency during fetal development, then a postnatal maturation stage of the GnRH pulse generator will have to be added to the schemata of female puberty.

An early influence of testicular androgen on the developing fetal brain seems to be a reasonable mechanism to account for this sex difference in hypothalamic function during infantile development, and this view is supported by the finding that male infants with partial androgen insensitivity due to mutations of the androgen receptor exhibit a female pattern of gonadotropin release in association with elevated testosterone levels.[214] However, the concomitant observation that, in subjects with complete androgen insensitivity, circulating concentrations of LH and testosterone are very low during the first 3 months of postnatal life[214] is counterintuitive. Parenthetically we consider that resolution of this intriguing paradox is likely to be particularly informative.

It is to be noted that during infantile development, the gametogenic functions of the ovary and testis are not activated despite the "adult like" gonadotropin drive to the gonad. In the infantile testis, Sertoli cells and undifferentiated type A spermatogonia proliferate, but the production of differentiating type B spermatogonia, the initial step in spermatogenesis, does not occur.[33,61,62,215,216] This deficit in seminiferous tubular function is likely to be accounted for by the absence of responsiveness of the Sertoli cell to androgen and FSH stimulation.[217] The precise age at which the primate gonad acquires the full capacity to respond to LH and FSH stimulation has not been established, but when it does, the infantile phase of robust gonadotropin secretion has been terminated and the hypogonadotropic state of the juvenile guarantees the quiescence of the primate testis for the remainder of prepubertal development.

Juvenile

Studies examining pulsatile profiles of circulating LH concentrations during juvenile development in both humans and monkeys (reviewed in detail in the previous edition of this chapter)[124] indicate that this mode of gonadotropin release, and presumable GnRH pulse generator activity, is not abolished in the juvenile, but rather is heavily suppressed to a variable degree depending on sex, and, perhaps, species. Indeed, it is now universally accepted that the marked reduction in gonadotropin secretion during juvenile development in primates is the result of a corresponding hiatus in robust pulsatile GnRH release from the hypothalamus.[218] In the premenarcheal rhesus monkey, spontaneous ovulatory menstrual cycles may be precociously induced by a chronic intermittent infusion of exogenous GnRH[21]; see Figure 32.2, and, in cognate studies of the male macaque, premature initiation of testosterone secretion and spermatogenesis has been reported.[148,219] In agonadal male rhesus monkeys between 15 and 18 months of age, which is the phase of development characterized by very low levels of LH and FSH (see below), the chronic intravenous intermittent infusion of GnRH elicits a prompt hypersecretion of both gonadotropins.[208,220,221] These results in the macaque are consistent with clinical findings that pulsatile GnRH treatment results in the full activation of the pituitary–gonadal axis in human subjects, with delayed puberty occasioned by defects in endogenous GnRH release.[149–151]

Although the juvenile stage of development is a hypogonadotropic phase, nighttime elevations in LH (and testosterone secretion) are observed in juvenile boys.[128,131,132] Nocturnal secretion of gonadotropin in juvenile boys is also reflected in urinary LH and FSH excretion.[222] Thus, it seems that the neuroendocrine mechanisms that underlie the nocturnal elevation in

[f]Note that several LH standards have been used to measure circulating LH concentrations shown in this and subsequent figures, so direct comparisons of specific concentrations across figures cannot be made.

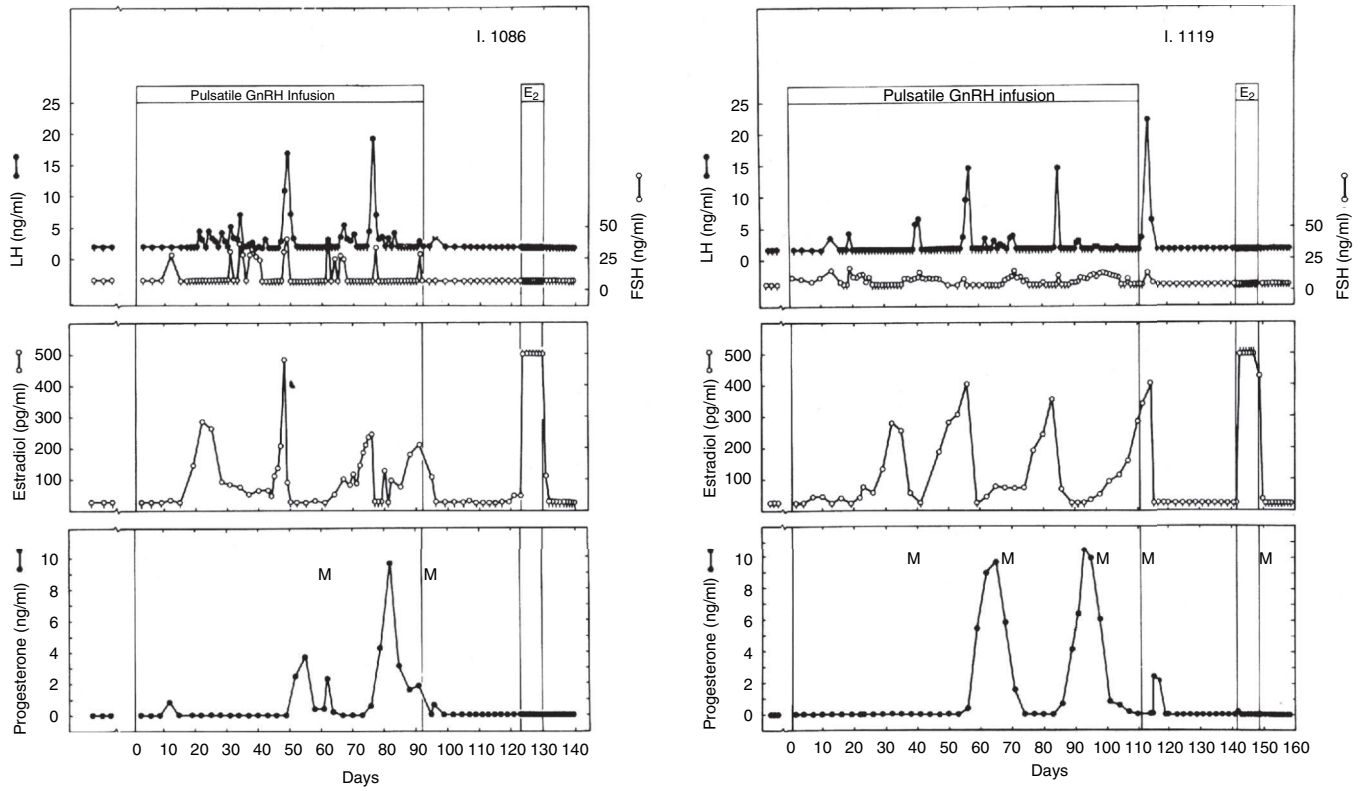

FIGURE 32.2 Ovulatory ovarian cycles in two premenarcheal rhesus monkeys induced by a chronic intermittent intravenous infusion of GnRH (1 pulse/h) initiated on day 0. Note that the pituitary–ovarian axis reverted to a prepubertal state following termination of GnRH treatment on days 92 and 111, respectively, and subsequent administration of estradiol (indicated by the unshaded bar labeled E2) failed to induce a gonadotropin surge. The occurrence of menstruation is indicated by M. *Source: Reprinted with permission from AAAS from Ref. 21.*

LH and testicular testosterone secretion during male puberty may be the same as those that are operative in infantile and juvenile primates. Studies of the agonadal female monkey indicate that the mechanisms for nocturnal modulation of hypothalamic GnRH release are also extant during infantile and juvenile development.[213,223]

Pubertal

During pubertal development there is a marked reaugmentation of pulsatile LH secretion that follows the reactivation of robust GnRH pulse generator activity, as directly demonstrated in one of the authors' laboratories by measuring pulsatile GnRH release in the region of the pituitary stalk–median eminence in the female monkey.[100,218,224]

Gonadotropin secretion during early human puberty is characteristically low during wakefulness and augmented in association with sleep, when large-amplitude LH pulses are observed (reviewed in detail in the previous edition of this book)[124]. Since marked circadian changes in pituitary responsivity to GnRH stimulation are unlikely, the large nocturnal LH pulses probably reflect high-amplitude GnRH discharges. The frequency of these discharges, originally observed only at night but later during the day, appears to increase noticeably from early to midpuberty in both boys and girls. The neuroendocrine basis of the nocturnal increase in LH release in pubertal boys, however, may differ from that in postpubertal males. In this regard, pulsatile LH secretion during the early hours of sleep in pubertal boys appears to be less responsive to the feedback action of testosterone,[225,226] and at this stage of development, in contrast to the situation in adults, the suppressive effect of testosterone on LH release that is observed appears independent of the endogenous opioids.[134,227] As is the case with the human male, the relationship between sleep and GnRH release appears to change with pubertal development in the female, with sleep inducing a deceleration in pulsatile LH release during the follicular phase of the human menstrual cycle.[228] An analogous situation appears to exist in the female monkey, since the GnRH pulse generator of the adult slows during the night.[229]

As puberty progresses, LH pulse frequency has been reported to decrease in both boys and girls.[230–233] In boys, this phenomenon has been attributed[231] to the action of rising testosterone concentrations to decelerate the frequency of the GnRH pulse generator. However, not all studies of LH pulse frequency during puberty in boys and girls have revealed the foregoing changes in the frequency of intermittent LH secretion.[234] Additionally,

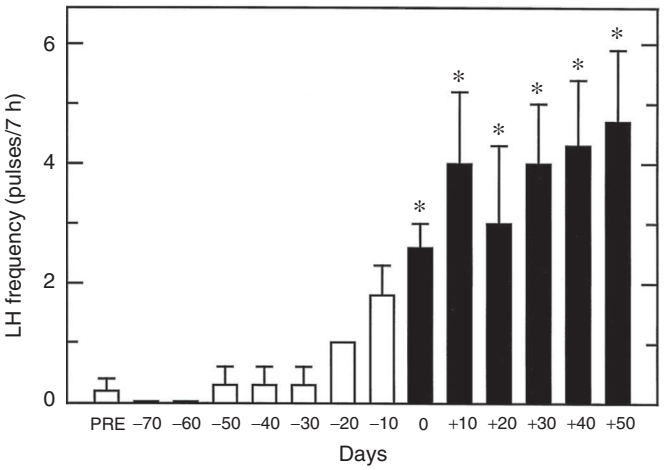

FIGURE 32.3 Peripubertal developmental changes in GnRH pulse generator activity, as reflected by nocturnal frequency of LH secretory episodes, obtained by aligning data for five agonadal male monkeys to the onset of the pubertal resurgence in pulsatile GnRH release (day 0). Heightened pituitary responsiveness to GnRH stimulation was maintained by a "priming" infusion of pulsatile GnRH between assessment windows. Ages of animals at day 0 ranged from 24 to 29 months. PRE, values for the earliest assessment window examined in each monkey, which ranged from 14 to 22 months of age; *asterisk*, significantly different from PRE ($P < 0.05$). *N* for each time point varies from 3 to 5. *Source: Reprinted with permission from Ref. 221.*

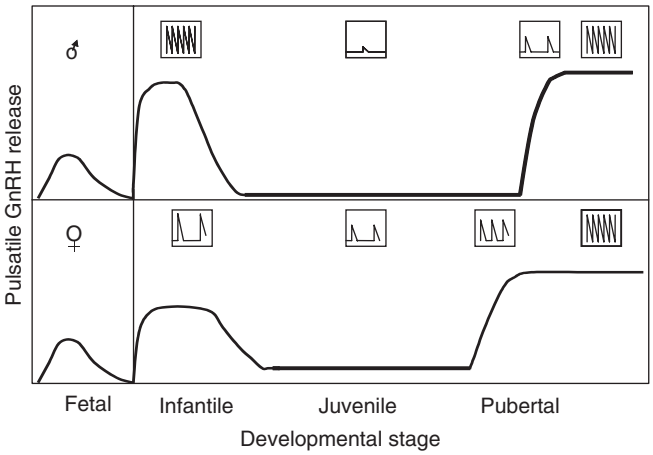

FIGURE 32.4 A model of the impact of the neurobiological brake on pulsatile GnRH release during juvenile development in males (above) and females (below). Insets indicate frequency of pulsatile GnRH release at respective stages of development. *Source: From Plant TM. Control of onset of puberty in primates.* Top Endocrinol *2002; **20** Chapterhouse Codex Ltd.*

direct measurement of hypothalamic GnRH release during puberty in the female monkey indicates that both frequency and amplitude increase at the time of puberty onset: from early to midpuberty the amplitude further increases, whereas the frequency remains constant.[218] Similar pubertal changes in the GnRH pulse frequency occur in the absence of the ovary.[224] Moreover, the pubertal increases in baseline GnRH release and GnRH pulse amplitude are greatly exaggerated in ovariectomized females compared to those in gonadally intact counterparts.[224] Longitudinal studies of agonadal male rhesus monkeys, in which pituitary responsiveness to endogenous hypothalamic GnRH discharges was heightened with a "priming" infusion of the synthetic decapeptide, have provided insight into the time course of the pubertal resurgence in pulsatile GnRH release in the absence of testicular steroid feedback.[221] As reflected by pulsatile patterns of LH secretion in such animals, nocturnal GnRH pulse generator activity accelerates explosively at the termination of the juvenile phase of development from <1 pulse/7 h to approximately 4 pulses/7 h over a period of less than 6 weeks (Figure 32.3). While this rapid tempo of the pubertal reawakening of the GnRH pulse generator in agonadal animals is presumably dampened in the normal situation by testicular inputs, it seems reasonable to propose that, in male primates, the acceleration of the GnRH pulse generator during puberty is an early and rapidly completed neurobiological event in the initiation of this phase of development.[221]

Indirect estimates of GnRH pulse amplitude during puberty have been made by comparing the amplitude of endogenous LH pulses with those elicited in the same pubertal subjects by bolus infusions of GnRH (previously shown to provide the pituitary with a hypophysiotropic stimulus comparable to that generated by the hypothalamus following puberty). Using this approach, two groups have concluded that GnRH pulse amplitude is similar in adult men and pubertal boys during sleep.[56,235] From the foregoing considerations it seems reasonable to conclude that the earliest detectable pubertal mode of operation of the GnRH pulse generator is composed of a high-amplitude, intermediate-frequency discharge at night that probably reflects an amplification of a comparable preexisting prepubertal mode of activity. As pubertal development continues, intermittent GnRH discharge occurs during the day, and the integrated 24 h frequency of the GnRH pulse generator accelerates. The time course of GnRH pulse generator activity during the mid- to postpubertal phase of development that is associated with increasing secretion of gonadal hormones is less clear.

A schematic representation of GnRH pulse generator activity from fetal development until the entry into adulthood in a representative male and female highly evolved primate is shown in Figure 32.4. The timing of primate puberty may therefore by conceptualized by the operation of two postnatal switches. The first is an "off" switch that is activated during infancy, resulting in the restraint of GnRH pulse generator activity that guarantees the gonadal quiescence of the child (human) and juvenile primate. The second is an "on" switch that leads to a reactivation of robust GnRH pulse generator activity and the onset of puberty.

GONADAL INDEPENDENCE OF THE POSTNATAL SWITCHES AND THE HYPOGONADOTROPIC STATE OF JUVENILE DEVELOPMENT

As described in the previous section, the postnatal pattern of gonadotropin secretion in highly evolved primates is characterized by elevated LH and FSH release during infancy and puberty (continuing into adulthood) separated by a relatively hypogonadotropic state during juvenile development. An important feature of this characteristic "primate" pattern of gonadotropin secretion postnatally is that its fundamental temporal aspects are preserved in the absence of the gonad. As shown in Figure 32.5, this feature of primate development is exemplified by the rhesus monkey rendered agonadal at birth by either ovariectomy or orchidectomy.[49,213] The non-gonadal reduction in LH and FSH secretion during juvenile development is also readily apparent from data obtained with agonadal boys and girls.[141,236–242] In the latter group of individuals, which comprise primarily phenotypically female subjects with Turner's syndrome, circulating LH concentrations that exist between 4 and 10 years of age are often in the range reported for normal children and are always well below values reported for postmenopausal subjects.

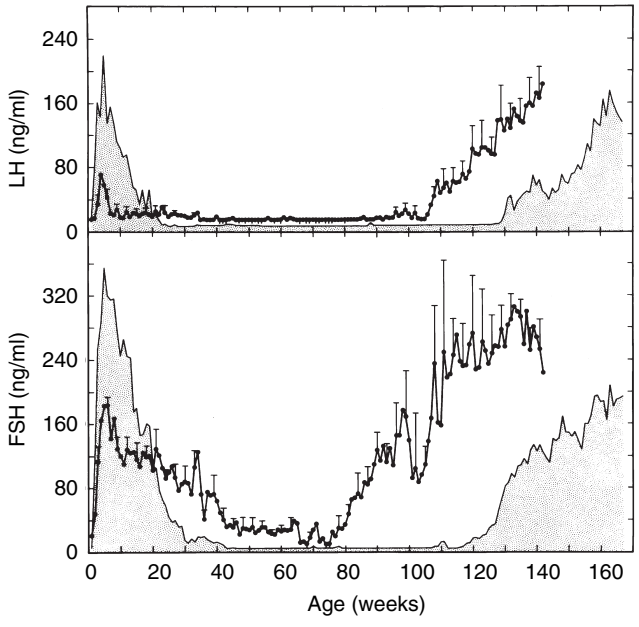

FIGURE 32.5 Time courses of circulating mean LH (top panel) and FSH (bottom panel) concentrations determined in blood samples collected in the morning from birth until 142–166 weeks of age in rhesus monkeys ovariectomized (•, $N=6$) and orchidectomized (*stippled area*, $N=4$) at 1 week of age. Note that the prepubertal hiatus in the secretion of FSH, and LH to a lesser extent, in agonadal females is truncated in comparison to that in castrated males. This difference between agonadal males and females, which presumably underlies the earlier onset of female puberty, is further exaggerated when nighttime concentrations of LH and FSH are examined (not shown). *Vertical bars* above data points indicate SEMs. *Source: The data for males are redrawn with permission, from Ref. 49.*

Taking the foregoing considerations together, it may be concluded that the low levels of circulating gonadotropins during juvenile development, which guarantee the relative quiescence of the gonad at this stage of primate development, are governed by a nongonadal brake, the effects of which are amplified, particularly in the female by inhibitory gonadal feedback. The site of action of the nongonadal brake is exerted at the level of the hypothalamus via GnRH release, and this issue will be explored in the next section.

While the nongonadal brake is waxing and waning during infancy and at the termination of the juvenile phase of development, respectively, inhibitory feedback actions of gonadal hormones on gonadotropin secretion, in both sexes, will play major roles in setting the quantitative level of FSH and LH secretion, and in modulating the precise duration of the hypogonadotropic juvenile state. Such feedback control during removal of the nongonadal brake at the termination of the juvenile phase of development will affect the tempo of gonadal maturation during puberty. For example, the negative feedback action of estradiol on gonadotropin release has been proposed as an important component of the neuroendocrine mechanisms that account for the temporal delay between menarche and first ovulation.[243–245] In this regard, it has been demonstrated for the rhesus monkey that the circulating estradiol concentrations required to prevent an elevation in gonadotropin following ovariectomy during the immediate postmenarcheal period are lower than those required to inhibit a postcastration hypersecretion of LH and FSH in adult females.[243,244] Similarly, in teenage girls with gonadal dysgenesis, the dose of conjugated estrogen required to suppress urinary excretion of LH and FSH into the normal range is less than that required for postmenopausal women.[246] Such steroid-imposed restraint on tonic gonadotropin secretion, which, according to studies of the pubertal monkey, is mediated at least in part by a hypothalamic site of action of estrogen to inhibit GnRH release,[224,247] is associated with, and is probably responsible for, the reduced efficacy of estrogen to exert its positive feedback action on gonadotropin secretion during this phase of pubertal development.[104,139] This latter view is consistent with the finding that estrogen-induced LH surges may be elicited in agonadal female monkeys at an earlier age than in intact animals.[248] Moreover, the onset of positive feedback in ovariectomized monkeys is observed in association with the initiation of the pubertal rise in tonic gonadotropin secretion.[248] Thus, it is reasonable to infer that ovulation does not generally take place immediately following menarche because the reawakening hypothalamic–pituitary unit is held in check by the relatively small amounts of estradiol produced by the ovary at this stage of development. A detailed discussion of these ideas

has been presented by Foster and his colleagues.[244] The operation of a similar feedback mechanism may also be invoked to account for the observation in the male rhesus monkey that the pubertal postcastration rise in LH and FSH secretion at 2 years of age precedes, by approximately 6 months, a detectable increase in testicular testosterone secretion in intact monkeys.[49]

In both man and the rhesus monkey, the degree and duration of the juvenile reduction in gonadotropin secretion are noticeably more marked in the male (Figure 32.5). The relatively weaker and shorter application of the agonadal brake or restraint on gonadotropin release during juvenile development in the female, which presumably underlies the earlier onset of puberty in this sex, is most clearly reflected by FSH secretion. In agonadal female monkeys and girls, plasma concentrations of this gonadotropin during the juvenile phase of development are consistently greater than those in normal age-matched subjects. As in infancy, the relatively high circulating FSH:LH ratio in the agonadal juvenile female is associated with a low LH pulse frequency.[223,238,240]

The sex difference in the degree to which gonadotropin secretion is restrained by the nongonadal brake during juvenile development is reinforced by studies in juvenile monkeys of the effect of castration on the subsequent pattern of LH and FSH secretion. Orchidectomy at this stage of development fails to elicit a postcastration rise in either LH or FSH.[75,249,250] Instead, plasma levels of these gonadotropins are maintained for many months at values that are indistinguishable from those of intact animals. Although the results of analogous studies in the prepubertal female are somewhat inconsistent,[75,213,223,243,249,251] it is now generally accepted that, in contrast to the male, ovariectomy of juvenile rhesus monkeys between 12 and 15 months of age results in a small postcastration increase in gonadotropin secretion, which is most marked at night and most noticeable for FSH.[213,223,251]

Before leaving the role of sex steroids in the developmental control of gonadotropin release, it is worth noting that the possibility that adrenal sex steroids exert an inhibitory action on LH and FSH release in the juvenile primates is, at best, remote. Bilateral adrenalectomy in agonadal infantile male rhesus monkeys at 1 month of age does not interrupt the prepubertal hiatus in gonadotropin secretion,[124,252] and human subjects with primary adrenal insufficiency do not exhibit precocious puberty.[3,9]

NEUROBIOLOGICAL BASES OF THE JUVENILE HIATUS IN PULSATILE GnRH RELEASE AND INITIATION OF PUBERTY

In contrast to what might be anticipated, there is little evidence to suggest that the ability of the GnRH neuronal network to respond to potent secretagogues, such as glutamate, is at all compromised in the juvenile hypothalamus. Trains of intermittent GnRH discharges with a periodicity and amplitude similar to those generated by the hypothalamus of the adult may be provoked from the brain of juvenile male monkeys in response to hourly stimulation with NMDA.[22,220,253] NMDA is an analog of the acidic amino acid glutamate, a major excitatory neurotransmitter of the mammalian CNS.[254] Although studies of NMDA action on the hypothalamus of the prepubertal female monkey have utilized only single bolus administration of the glutamate receptor agonist,[255,256] repetitive stimulation of the stalk–median eminence region of the juvenile female monkey electrically or by direct application of an α_1-adrenergic receptor agonist will also elicit a train of GnRH discharges.[257,258]

These physiological findings are largely consistent with those describing GnRH mRNA and peptide content. In the agonadal male macaque, hypothalamic GnRH content as estimated by bioassay and RIA remains unchanged throughout postnatal life.[250,259] Moreover, in the rhesus monkey and some other species of primate, the distribution of GnRH neurons and their peptide content, as reflected by immunohistochemical analysis, are comparable in infant, juvenile, and adult animals,[260–266] Figure 32.6. The data on GnRH mRNA levels are less consistent. In the agonadal male rhesus monkey, the levels of this transcript as determined by RNAse protection are indistinguishable in the MBH of infant and juvenile animals,[267] but at the time of the pubertal resurgence of GnRH release, such animals show a modest increase in this mRNA.[250] In the intact male macaque, the pubertal increase in *GnRH* expression has been reported to be either exaggerated (qPCR)[268] or absent (in situ hybridization).[269] Although in one study of female monkeys, GnRH mRNA levels in the MBH of juvenile and pubertal aged animals were reported to be similar,[270] in another a robust increase in this mRNA has been noted during the juvenile—pubertal transition (Terasawa et al., unpublished observation).

These findings suggest that GnRH neurons in the juvenile hypothalamus are endowed with the molecular machinery required for generating an intermittent discharge of the peptide. Moreover, this molecular machinery may be prematurely activated with apparent ease either experimentally[22] or pathophysiologically, as in GnRH-dependent precocious gonardarche.[3,9,143,144,154] The biosynthetic properties of hypothalamic GnRH neurons in the juvenile may be contrasted with those of the gonadotroph at a comparable stage of development (Figure 32.7). In most instances, the magnitude of biosynthesis of a given secretory product is strongly coupled to the degree of cellular activation.[271] Thus, as expected in the absence of a GnRH drive during the juvenile phase of development, LH content of the gonadotroph and circulating levels of LH are at least an order of magnitude

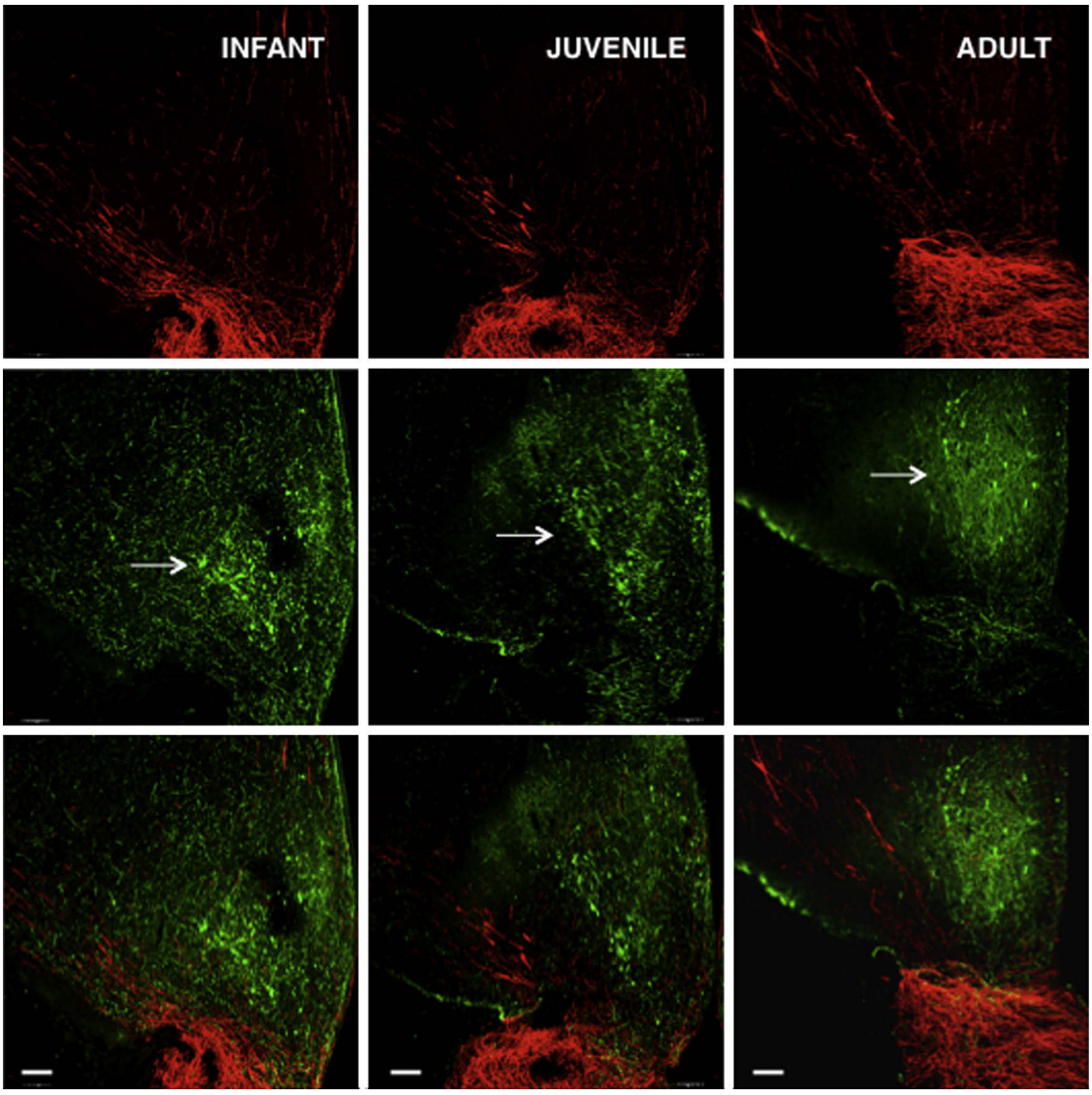

FIGURE 32.6 This figure is reproduced in color in the color plate section. Confocal images illustrating developmental changes in GnRH (top panels) and kisspeptin (middle panels) neurons (immunopositive perikarya and fibers) in the MBH at the mid-tuberal level during postnatal development in the agonadal male rhesus monkey. The lower panels show the merged images. A hemi-hypothalamic section is shown for an infant (left-hand panels), juvenile (center panels), and adult (right-hand panels) animal; stages of postnatal development corresponding to the on-off-on pattern of GnRH pulse generator activity in primates. Note that kisspeptin immunoactivity is reduced in the juvenile MBH at a time when GnRH pulsatility is arrested but GnRH immunoactivity in the median eminence is maintained during this phase of development. In most cases the ependymal lining of the third ventricle is visible on the right-hand boundary of each hemi-section. *Arrow* indicates kisspeptin neurons in the arcuate nucleus. Scale bar, 100 μm. *Source: Parts of this montage have been reprinted with permission from Refs 170,266.*

lower than those observed in the infant or adult.[259] In addition, the mRNA levels encoding the LHβ and FSHβ gonadotropin subunits are low (Winters and Plant, unpublished observations), and the pituitary requires weeks of intermittent GnRH stimulation before an adult-like pattern of gonadotropin secretion is elicited.[221] From these data, it is reasonable to posit that (1) the GnRH neurons in the hypothalamus of the juvenile monkey are receiving stimulatory afferent input (thereby maintaining the cell's biosynthetic potential), and (2) the GnRH pulse generating system that leads to the intermittent discharge of the peptide into the hypophyseal portal circulation during infancy and puberty is restrained during juvenile development.

Kisspeptin

As described previously, compelling evidence is emerging to indicate that the output of the hypothalamic GnRH pulse generator is relayed to the GnRH neuronal network by an intermittent kisspeptin signal generated by KNDy neurons in the arcuate nucleus. That intermittent kisspeptin release in the region of the median eminence is attenuated during juvenile development in the

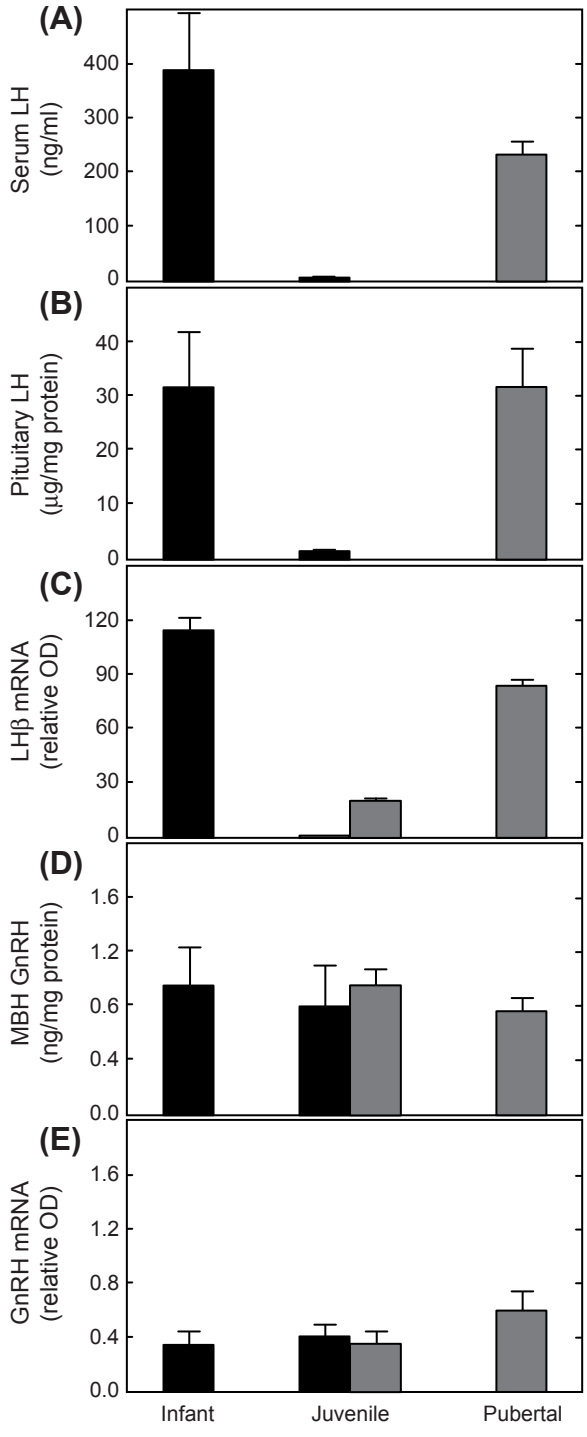

FIGURE 32.7 The hypogonadotropic state of the agonadal juvenile male monkey in comparison with that of the infant and pubertal state (panel A) is associated with a correspondingly low pituitary LH protein and LHβ mRNA content (panels B and C, respectively) but, interestingly, the content of GnRH peptide and mRNA in the MBH at this stage of development is maintained (panels D and E, respectively). Two histograms are shown for the juvenile stage of development because two separate groups of animals at this stage of development were studied: one was used in a study comparing infants and juveniles[267] (closed histogram) and one was used to compare juvenile and pubertal monkeys[250] (gray histogram). Means and SE are shown. *Source: Data for serum LH concentration, pituitary LH content, MBH GnRH peptide, and mRNA content redrawn from Refs 250, 259, 267. Data for LHβ; Winters SJ, Plant TM, unpublished observations.*

female monkey has been directly demonstrated[272,273] (Figure 32.8). Application of microdialysis to sample peptide release in the median eminence revealed that discharges of kisspeptin in this region of the hypothalamus during juvenile development were of low amplitude and occurred with a long interpulse interval, a pattern of release that contrasted to high amplitude and frequency release of kisspeptin in pubertal monkeys. This developmental change in pulsatile kisspeptin release was observed in both intact and ovariectomized females[273] and was similar to that of GnRH release in this region of the basal hypothalamus.[218,224] Importantly, the frequencies of kisspeptin discharges at the two stages of development were independent of the ovary. An impact of the ovary on kisspeptin release, as to be expected, was evident in the pubertal monkey, where both pulse amplitude and mean release of kisspeptin were markedly increased by ovariectomy, presumably due to loss of negative feedback from ovarian estradiol. Indeed, replacement of estradiol in pubertal ovariectomized animals suppressed kisspeptin release, while in prepubertal monkeys kisspeptin release was not affected by estradiol replacement.[273] Thus, peripubertal changes in ovarian steroid regulation of kisspeptin release are similar to those seen with GnRH release.[247] The attenuation in pulsatile kisspeptin release in the median eminence in the juvenile monkey is associated with a low level of *KISS1* expression in the MBH (presumably in the arcuate nucleus) in ovarian intact female and agonadal male monkeys.[274]

The changes in *KISS1* expression during the juvenile–pubertal transition in the agonadal male is paralleled by changes in the peptide (Figure 32.6).

Studies of KNDy neurons in the infant and of how their function changes in the transition to the juvenile stage of development when robust activity of the GnRH pulse generator is being brought into check by the "off" switch are scant. However, a recent study of the agonadal male monkey has shown that the number of immunopositive kisspeptin neurons in the MBH is greater in the infant than in the juvenile, thus paralleling the decline in GnRH pulsatility during this developmental transition,[266] Figures 32.6 and 32.9. The latter result is consistent with the notion that the reduction in pulsatile GnRH release during the infantile–juvenile transition may be causally related to a decrease in the kisspeptin output of the GnRH pulse generator, an idea further supported by the finding from human genetics that an infant boy with loss of function mutation in KISS1R was reported to be hypogonadotropic.[275] Taking the foregoing considerations together, it seems reasonable to propose that the neurobiology underlying the switch that leads to the attenuation of pulsatile GnRH release during the transition from infantile to juvenile is thrown into reverse at the termination of the juvenile phase of development.

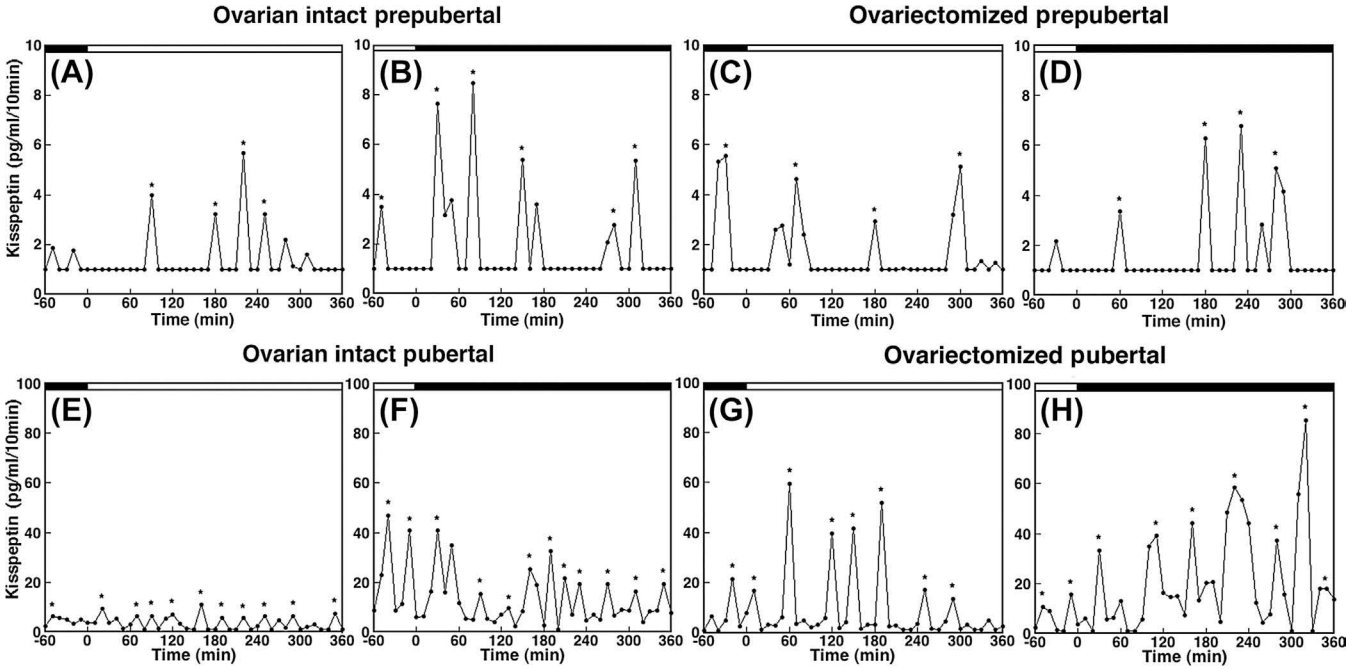

FIGURE 32.8 Pulsatile kisspeptin release in the region of the pituitary stalk and median eminence of ovarian intact prepubertal (panels (A) and (B)) and pubertal (panels (E) and (F)) monkeys, and ovariectomized animals at similar stages of development (panels (C) and (D), and (G) and (H), respectively) as assessed by microdialysis. Note that the scale on the *y*-axis in panels (E)–(H) (pubertal monkeys) is 10-fold higher than that in panels (A)–(D) (prepubertal monkeys). The dark phase of the light–dark cycle is indicated on the top of each of the eight panels with a dark solid line. *Asterisks* indicate increments in kisspeptin identified as pulses by PULSAR. *Source: Modified with permission from Ref. 273.*

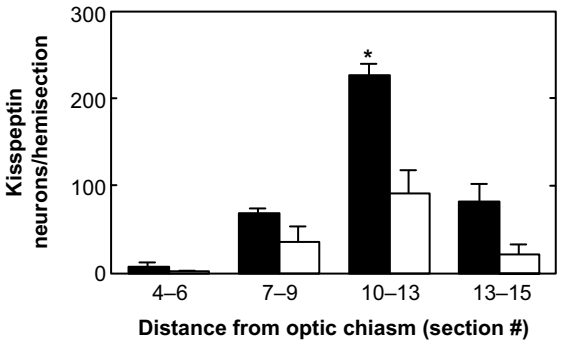

FIGURE 32.9 The number of kisspeptin neurons per hemi cross-section throughout the arcuate nucleus of the agonadal infant male monkey (closed histograms) is greater than that in juveniles monkeys of similar gonadal stature (open histograms). Section number indicates distance posterior from the optic chiasm with section 1, the first retrochiasmatic section. The distance between two sequentially numbered sections was 250 μm. * $p < 0.05$. *Source: Reprinted with permission from Ref. 266.*

That an attenuated kisspeptin output from the GnRH pulse generator is the proximal cause of diminished GnRH release in the juvenile is also supported by the finding that in the agonadal juvenile male monkey (with a GnRH primed pituitary), repetitive stimulation of the GnRH neuronal network with hourly intravenous injections of kisspeptin is able to induce a premature and sustained train of LH discharges similar to that produced by spontaneous GnRH pulse generator activity in pubertal animals,[276] Figure 32.10. It is important to note that in this model the kisspeptin-induced train of LH pulses is abolished by concomitant administration of a GnRH receptor antagonist, confirming a hypothalamic action of kisspeptin.[276] Dose-dependent kisspeptin-induced GnRH release in the median eminence of the juvenile female monkey has been directly demonstrated using microdialysis,[277] further suggesting a causal relationship between the reduced kisspeptin output and low levels of GnRH release during this phase of prepubertal development.

From the foregoing discussion it is apparent that the critical role of kisspeptin in the initiation of puberty can be accounted for entirely by the role of this neuropeptide in mediating the output of the GnRH pulse generator. This realization led us to recently propose a new model for the control of the timing of puberty in primates.[168] According to this model (Figure 32.11), kisspeptin neurons in the arcuate nucleus play no regulatory role in dictating the timing of puberty. Rather, as a component of the hypothalamic GnRH pulse generator, they are a slave to the upstream regulatory mechanisms that determine puberty onset.

Expression of *KISS1R* by GnRH neurons has been demonstrated in non-primate species using in situ hybridization,[278] and therefore the question of whether changes in KISS1R signaling in GnRH neurons also contribute to the hiatus in kisspeptin tone to the GnRH neuronal network during juvenile development merits

consideration. KISS1R mRNA levels in the MBH increase during puberty in the intact female monkey, although this was not observed in the agonadal male[274] and studies of expression of this receptor specifically by primate GnRH neurons have not been conducted. Nevertheless, kisspeptin-induced GnRH release in the female monkey is larger in pubertal animals than in juveniles,[277] indicating that an increase in responsiveness of the GnRH neuronal network to kisspeptin stimulation contributes to the pubertal resurgence of pulsatile GnRH release.

Amino Acid Neurotransmitters

γ-Aminobutyric acid (GABA) and glutamate are the major inhibitory and stimulatory neurotransmitters, respectively, in the CNS,[279] and both appear to be intimately involved in the regulation of the postnatal pattern of pulsatile GnRH release.

As described previously, sustained trains of GnRH-induced LH discharges may also be elicited from the MBH of the juvenile male by repetitive activation of hypothalamic NMDA receptors.[253] Moreover, the time course of these LH discharges is essentially identical to that induced by intermittent intravenous infusion of kisspeptin,[276] and, as with kisspeptin, hypothalamic glutamate release increases at the time of puberty in the female rhesus monkey[280]; Figure 32.12. Furthermore, a preliminary study indicates that the competitive NMDA receptor antagonist, DL-2-amino-5-phosphono-pentanoic acid, can interrupt GnRH pulse generator activity in the adult monkey.[281] The exact relationship between the excitatory amino acid and the neuropeptide in the context of GnRH pulse generation, however, remains to be elucidated. Although non-NMDA receptors, and in particular kainate receptors, are most highly expressed in the region of the arcuate nucleus/median eminence in the monkey (T.M. Plant, unpublished data), intermittent stimulation of the juvenile hypothalamus with kainic acid, in contrast to that with NMDA, fails to sustain a train of GnRH-induced LH discharges.[282]

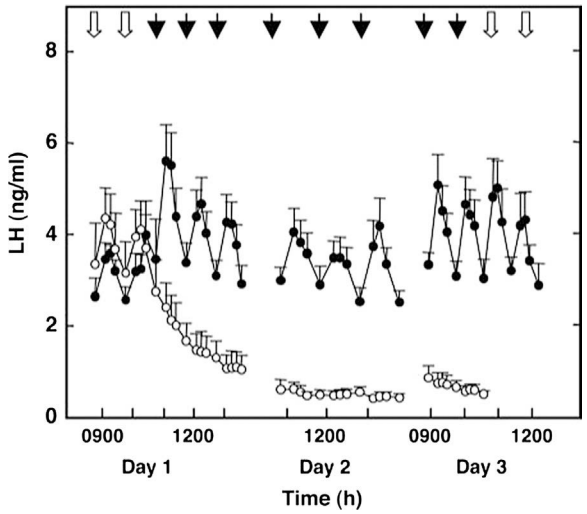

FIGURE 32.10 LH responses in agonadal GnRH primed juvenile male rhesus monkeys ($N=4$) during the last two priming infusions of GnRH (administered on Day 1 at 0900 and 1000 h, open arrows) and during brief hourly intravenous infusions of either kisspeptin or vehicle (black arrows) initiated on Day 1 at 1100 h and maintained for 48 h. The response to the experiment in which kisspeptin was administered is shown by the black data points. Note that although the kisspeptin and vehicle injections were administered without interruption for 48 h, only those injections to which the LH response was monitored are indicated. The LH response to the first two repriming pulses of GnRH are shown for the kisspeptin experiment (administered on Day 3 at 1100 and 1200 h, open arrow). The priming infusion before and after the kisspeptin infusion produces a pulsatile discharge of LH comparable to that observed spontaneously in pubertal animals. The response to repetitive kisspeptin administration was abolished by concomitant treatment with a GnRH receptor antagonist (data not shown), indicating the intermittent kisspeptin infusion provides the GnRH network of the juvenile hypothalamus with a stimulus similar to that produced endogenously by the GnRH pulse generator in pubertal animals. Vertical lines above data points indicate SEM. *Source: Reprinted with permission from Ref. 276.*

In the case of GABA, a series of studies of the female monkey has provided unequivocal evidence that this amino acid plays a critical role in maintaining the suppression of pulsatile GnRH release throughout juvenile development.[100] In one study, chronic repetitive administration of bicuculline (a $GABA_A$ receptor antagonist) into the base of the third cerebroventricle in the juvenile female led to precocious puberty, as reflected by premature menarche and first ovulation.[283] That this precocity was the result of premature and sustained GnRH pulsatility is supported by the following studies. Employing push–pull perfusion or microdialysis of the stalk–median eminence to quantitate neuropeptide and neurotransmitter release in the hypothalamus of the female monkey, the pubertal rise in GnRH secretion and, interestingly, the associated increase in glutamate release were demonstrated to be temporally coupled to a decrease in GABA release in this region of the brain.[280,284] Moreover, an acute reduction of GABA signaling in the stalk–median eminence region of the juvenile monkey induced by local administration of bicuculline, or antisense oligodeoxynucleotide for the mRNA encoding the GABA-synthesizing enzyme, glutamic acid decarboxylase 67 (GAD 67), elicited an immediate discharge of GnRH,[284,285] which was associated with an increase in glutamate release.[280] More recently, similar bicuculline injections to juvenile female monkeys have been shown to induce kisspeptin release,[286] Figure 32.13. In contrast, bicuculline infusion into pubertal female monkeys failed to stimulate either GnRH or kisspeptin release.[284,286] The elevated levels of GABA release in the juvenile hypothalamus of the female are associated with an increase in GABA synthesis, as infusion of antisense oligodeoxynucleotide for the mRNA encoding GAD 67 into the median eminence greatly stimulates GnRH release in juvenile monkeys,

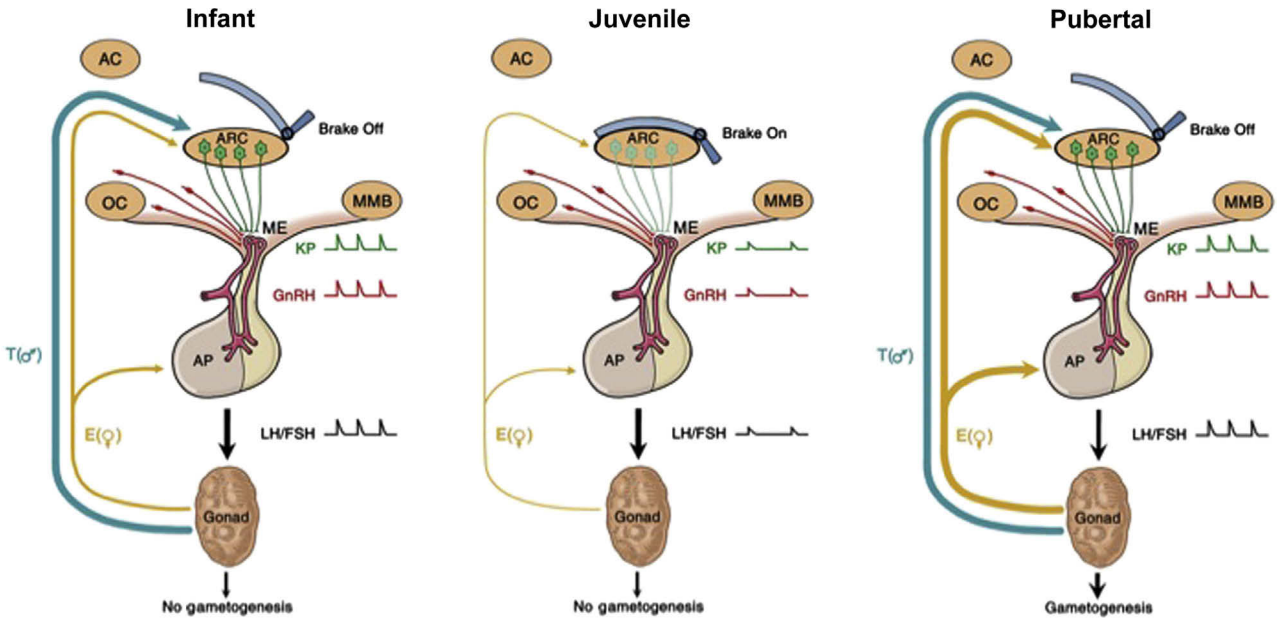

FIGURE 32.11 A model for the control of the timing of puberty. The role of kisspeptin signaling is posited to be a critical component of the neural machinery that generates pulsatile gonadotropin-releasing hormone (GnRH) release from the hypothalamus (the hypothalamic GnRH pulse generator). In this model, the hypothalamic GnRH pulse generator resides in the arcuate nucleus (ARC) and the output of the pulse generator is relayed to GnRH terminals in the median eminence (ME) by kisspeptin projections arising from KNDy neurons in the ARC. During infancy (*left panel*), GnRH pulse generator activity is robust, leading to intermittent release of kisspeptin in the ME, resulting in a corresponding pattern of GnRH release into the portal circulation. This, in turn, drives pulsatile gonadotropin secretion. In the transition from infancy to the juvenile phase of development (*middle panel*), a neurobiological brake restrains the GnRH pulse generator and pulsatile release of kisspeptin in the ME is markedly suppressed. This leads to reduced GnRH release and to a hypogonadotropic state in the juvenile period. Puberty is triggered when the neural brake is released and GnRH pulse generator activity with robust intermittent release of kisspeptin in the ME is reactivated (*right panel*). According to this model, the mystery of primate puberty lies in the nature of the neurobiological brake and in the mechanism that times its application during infancy and its release at the end of the juvenile phase of development. It should be noted that the ability of the postnatal gonad to respond fully to gonadotropin stimulation is not acquired until the juvenile stage of development, by which time luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion is low as a result of the GnRH pulse generator being brought into check. The thickness of the arrows (T, testosterone and E, estradiol) from gonad to ARC indicating negative feedback by the testis and ovary, respectively, reflects the degree of gonadal steroid inhibition exerted on LH secretion at these three stages of development. AC, anterior commissure; AP, anterior pituitary gland, ARC, arcuate nucleus; OC, optic chiasm; ME, median eminence; MMB, mammillary body. *Source: Reprinted from Ref. 168.*

whereas the same antisense oligodeoxynucleotide infusion in pubertal monkeys results in significantly smaller GnRH increase.[285,287] Importantly, there is a temporal relationship between the decrease in GABA and the increase in glutamate when antisense oligodeoxynucleotide is infused into the median eminence.[280] Specifically, a decrease in GABA release in the stalk–median eminence triggers a GnRH discharge with a subsequent increase in glutamate release in this hypothalamic region that further contributes to the pubertal increase in GnRH. In the male monkey, on the other hand, it is unclear whether GABA inhibition is responsible for the juvenile hiatus of GnRH release, as equivalent experiments have not been conducted and the expression of GAD 65 and GAD 67 mRNA[250,288] does not change across puberty. In the infant–juvenile transition during the attenuation in GnRH pulse generator activity, however, GAD 65 and GAD 67 mRNA levels increase in the male hypothalamus.[267]

Additional support for a role of GABA comes from clinical observations in which administration of loreclezole, a GABA agonist, to an 11-month-old girl with epilepsy and precocious gonadarche, led to decreased LH secretion and regression in pubertal development.[289] Pubertal delay has also been reported in epileptic boys treated with valproic acid, a drug with GABAergic activity.[290] It should be noted, however, that the pharmacology of valproic acid is complex, and others have reported that this drug, like other antiepileptic agents, does not invariably delay sexual maturation.[291]

While the foregoing considerations provide compelling evidence in support of the view that both GABA and glutamate play an important role in dictating the kisspeptin output of the GnRH pulse generator during juvenile development and the transition into puberty in females, it is likely that additional regulatory factors are yet to be identified. In this regard, the induction of precocious menarche in response to repetitive bicuculline administration to juvenile female monkeys (see above) appears to be related to the age at which GABA antagonism is initiated: the younger the age the longer

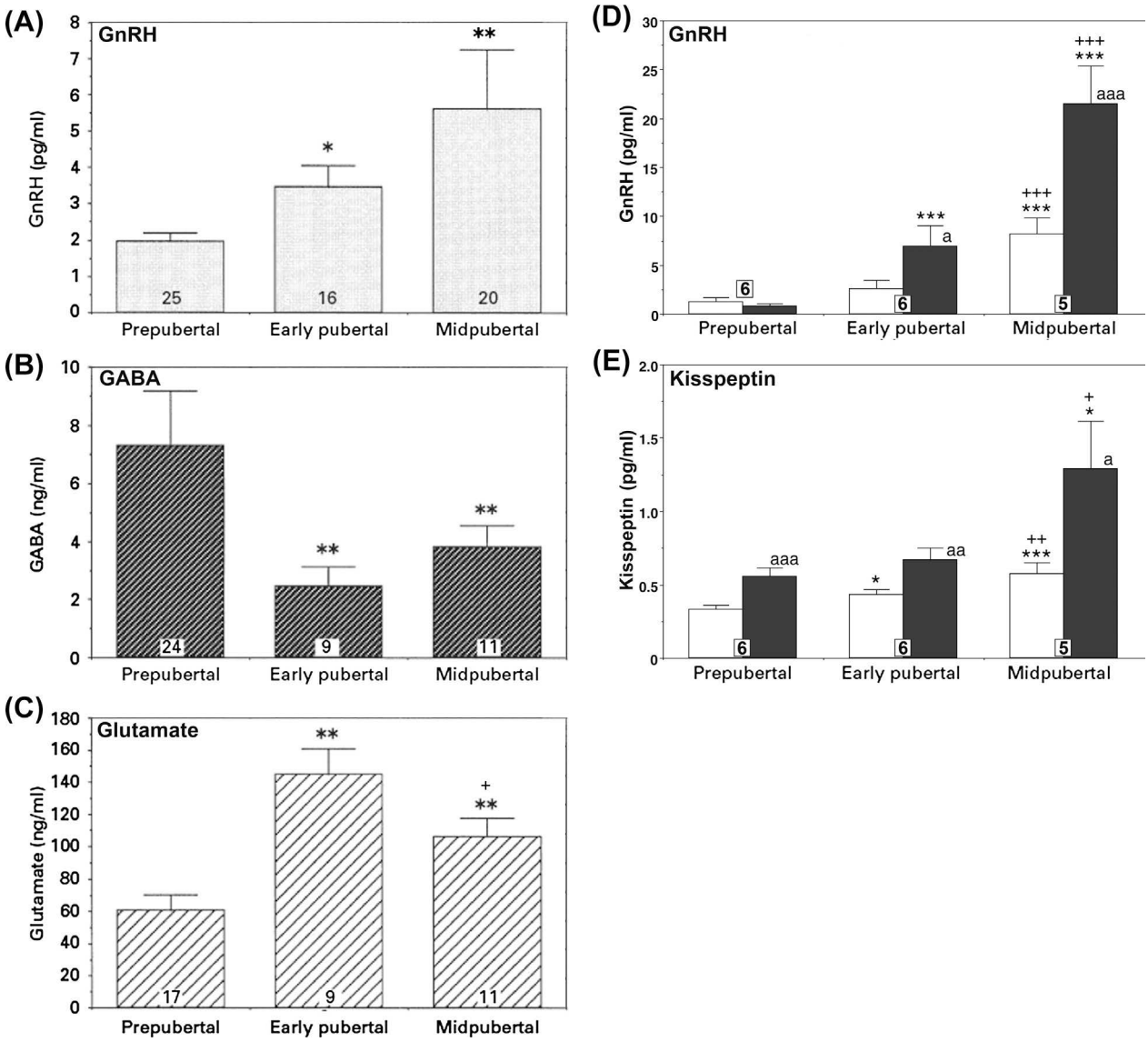

FIGURE 32.12 Release of GnRH (A) and (D), GABA (B) glutamate (C), and kisspeptin (E) in the stalk–median eminence of ovariectomized pre-, early- and mid-pubertal female rhesus monkeys, as measured in hypothalamic perfusates. GnRH (A), GABA (B), and glutamate (C) were measured in the same samples obtained in one study[280] and GnRH (D) and kisspeptin (E) were measured in the same samples obtained in another study.[272] In the first study (A)–(C), samples were collected in the morning only, whereas in the second study (D and E), samples were collected in the morning (open bars) and evening (closed bars). *$p<0.05$ versus prepubertal; **$p<0.01$ versus prepubertal; ***$p<0.001$ versus early pubertal; +$p<0.05$ versus prepubertal; ++$p<0.01$ versus early pubertal; +++$p<0.001$ versus early pubertal; a $p<0.05$ versus AM; aa $p<0.01$ versus Am, aaa $p<0.001$ versus AM. $N=5$–25 (see individual bars). *Source: Reproduced with permission from Refs 272,280.*

the duration to precocious menarche (Terasawa, unpublished observations).

Neuropeptide Y

Evidence obtained primarily with the male rhesus monkey indicates that neuropeptide Y (NPY) may be an important component of the neurobiological brake responsible for restraining pulsatile GnRH release during juvenile development.[250] First, changes in hypothalamic *NPY* expression and NPY content during the transition from the infant to the juvenile and to the pubertal phase of development in agonadal male monkeys are inversely related to those in pulsatile GnRH release; namely *NPY* expression is elevated during the juvenile phase of development and low in association with robust GnRH pulse generator activity during infancy and at puberty. Second, a marked inhibition of pulsatile GnRH release is observed in agonadal postpubertal male and female monkeys following central administration of NPY.[292–294] Although the inhibition of GnRH release in the postpubertal situation is mediated, at least in part, by the NPY

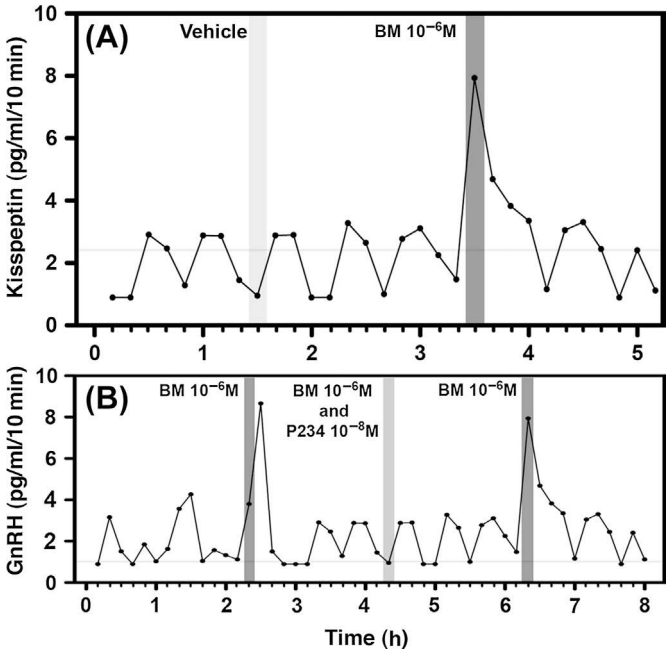

FIGURE 32.13 Panel (A): A brief 10 min infusion of the $GABA_A$ antagonist, bicuculline (BM) into the stalk–median eminence region (dark shaded vertical bar) stimulates kisspeptin release in a prepubertal female monkey. Panel (B): Coinfusion of the kisspeptin receptor antagonist, peptide 234 (P234), and BM (light shaded vertical bar) blocks the action of the $GABA_A$ antagonist and elicits GnRH release. *Source: Reprinted with permission from Ref. 286.*

Y1 receptor, the pharmacological data supporting a role for this receptor sub-type in the prepubertal attenuation of pulsatile GnRH release are tenuous.[292] Also, the search for inactivating mutations of the NPY Y1 receptor gene in children with GnRH dependent precocious gonadarche (see below) has not been successful.[295,296]

In addition to the posited inhibitory action of NPY on GnRH release during juvenile development (discussed previously), this neuropeptide has also been implicated in the stimulation of GnRH release at the time of puberty and in adulthood. In both pubertal and postpubertal female monkeys, but not in prepubertal subjects, NPY infusion in the region of the median eminence stimulates GnRH release,[294,297,298] and infusion of NPY antibody or antisense oligodeoxynucleotide for NPY mRNA in this hypothalamic area suppresses GnRH release.[297,299] The pubertal emergence of this stimulatory action of NPY on GnRH release is associated with an amplification of pulsatile release of NPY in the median eminence that is temporally coupled to that of GnRH.[297,300] The most parsimonious explanation to account for the paradox of the inhibitory and excitatory actions of NPY on GnRH release is to posit two subsets of NPY neurons.[301] One subset comprises a component of the brake that holds the GnRH pulse generator in check during juvenile development. The other subset comprises a component that appears to be related to GnRH pulse generation and is only manifest after the juvenile brake on the GnRH pulse generator has been lifted. Whether these two putative subsets of NPY perikarya are anatomically distinct has not been examined.

Opioid Peptides

The opioid peptides were the first to attract attention as potential candidates responsible for the attenuation of pulsatile GnRH release in the juvenile because of their established inhibitory action on GnRH secretion in sexually mature monkeys and human beings,[302–306] and because endogenous opioid peptides are known to exert presynaptic inhibition in the magnocellular system.[307] Administration of opioid antagonists such as naloxone and naltrexone, however, has consistently failed to release the prepubertal brake on the GnRH pulse generator as reflected by the failure of these drugs to elicit LH secretion in prepubertal children,[308–311] chimpanzees,[312] and rhesus monkeys.[255,313,314] The failure to detect naloxone-induced GnRH release during the prepubertal period cannot be accounted for by the decreased sensitivity of the gonadotrope during this phase of development since, in studies of the monkey, the responsivity of these pituitary cells to GnRH was greatly heightened prior to the studies with exogenous GnRH.[313] Additionally, puberty in the male monkey appears to be associated with an up-regulation of the POMC gene.[269] Consistent with this report, preliminary data in female monkeys indicate that β-endorphin release in the stalk–median eminence region increases in association with puberty onset.[315]

Other Neuronal Factors

Peripheral intravenous administration of cholecystokinin (CCK), like that of kisspeptin is also able to elicit precocious GnRH release in the agonadal juvenile male monkey.[316] In the context of the timing of the pubertal resurgence in GnRH release, this action of CCK, which is associated with a generalized activation of the neuroendocrine hypothalamus, has not been further examined. Finally, acute central administration of methoxamine, an α_1-adrenergic receptor agonist, can elicit a GnRH discharge in the premenarcheal rhesus monkey,[258] whereas intravenous injection of the α-adrenergic receptor antagonist phentolamine blocks GnRH pulsatility in the adult female.[317,318]

Glial-Derived Growth Factors

Glial-derived growth factors, such as transforming growth factor (TGF) α, neuroregulins, and their cognate ERBB transmembrane tyrosine kinase receptors, play a role in the timing of puberty in the rat (see Chapter 30).

The actions of these growth factors on GnRH neurons are stimulatory and indirect, involving autocrine/paracrine actions of factors such as prostaglandin E2 released from neighboring astrocytes. Studies of the monkey in this regard are limited: TGFα mRNA in the hypothalamus of the female rhesus monkey is increased dramatically at the time of puberty,[270] although in the agonadal male expression of this gene is only marginally increased at a corresponding stage of hypothalamic development.[250] Further support for the view that glial factors may play a role in the pubertal resurgence in GnRH release in highly evolved primates is provided by findings that hypothalamic lesions result, on the one hand, in an astrocytic response that includes activation of TGFα and expression of ERBB receptors,[319,320] and, on the other hand, may be associated with precocious gonadarche in children[321–323] and in juvenile monkeys.[324] Thus, precocity in primates associated with hypothalamic lesions, and in children who have been subjected to chemotherapy or cranial irradiation, or who have sustained head trauma,[321–323] may result from increased ERBB signaling rather than by interruption of inhibitory inputs to the GnRH network, as was previously proposed.[325] Additionally, hamartomas removed from two patients with precocious gonadarche have been found to be rich in astrocytes containing TGFα, but devoid of GnRH neurons,[325] again indicating a role of increased ERBB signaling in the etiology of this precocity. These arguments, which have been primarily articulated by Ojeda and his colleagues,[325] are consistent with the finding in the rat that infusion of an ERBB receptor antagonist at the site of the hypothalamic lesion prevents the puberty-advancing action of the lesion.[319]

Structural Remodeling

Regardless of the relative importance and identities of transynaptic and astroglial systems that underlie the neurobiological brake on the GnRH pulse generator during juvenile development, it is reasonable to propose that this brake will involve a reorganization of the anatomic substrate that governs the control of the GnRH pulse generator. The capacity for structural plasticity within the mammalian brain appears to be associated with the expression of the "embryonic" or polysialic acid—neural cell adhesion molecule,[326] and a study of the rhesus monkey employing immunocytochemical and immunoblot analysis has demonstrated that this cell surface glycoprotein is expressed in the region of the arcuate nucleus and median eminence.[327] Although it is not known whether the arcuate nucleus is remodeled during postnatal development in primates, quantitative ultrastructural studies have demonstrated that synaptic input to GnRH perikarya in the MBH of the agonadal male monkey declines in association with the pubertal resurgence in pulsatile GnRH release. Interestingly, this structural remodeling during peripubertal development in the monkey has only been observed in the MBH.[328] On the other hand, the degree of glial ensheathment of GnRH neurons in the MBH of agonadal monkeys did not appear to be developmentally regulated,[328] although peripubertal changes in glial ensheathment have been reported in the preoptic area in the female rhesus monkey.[329]

It will be obvious from the foregoing discussion of the neurobiological basis of the juvenile hiatus in GnRH pulse generator activity that our understanding of this phenomenon is at best fragmentary. Moreover, in some cases, for example, that of the role of GABA, studies have been restricted primarily to the female, while in others the male has been the primary sex examined. As such, it is not possible to provide a useful summary of either (1) the relative importance of various neurotransmitters, neuropeptides, and glial factors underlying the hiatus, or (2) whether there are qualitatively sex differences in the neural mechanisms utilized to achieve the hiatus.

THEORETICAL PHYSIOLOGICAL CONTROLS GOVERNING THE DURATION OF THE PREPUBERTAL HIATUS IN GnRH SECRETION

Two theoretical physiological control systems may be invoked to account for the prepubertal hiatus in GnRH pulse generator activity in highly evovled primates. One involves the concept of a central neural timekeeping mechanism able to measure age and programmed to operate a gating system that would interrupt GnRH pulse generator activity from infancy to puberty. The other model requires positing a central neural mechanism capable of tracking one or more hormonal signals that reflect somatic size/growth/maturation.[330] Such a somatometer would, in turn, govern the postnatal ontogeny of GnRH release in much the same fashion proposed for the first model of a pubertal clock.

Attempts to examine the anatomic location of such putative control systems have been made using a classical technique known as surgical deafferentation, whereby the MBH is isolated from the rest of the brain and the impact on the pituitary–gonadal axis is monitored. When this procedure was performed on juvenile female monkeys, the age at the onset of puberty was similar to that of control monkeys.[331,332] In contrast to surgical deafferentation of the MBH in adults,[333] however, the diurnal variation in cortisol secretion was not abolished in the pubertal animals,[331,332] suggesting that isolation of the GnRH network from extrahypothalamic inputs may have been incomplete. Thus, it is premature to conclude that the entire neural component of the puberty control system resides within this area of the hypothalamus.

Somatometer

In considering a somatometer, it has been long viewed as axiomatic[334] that growth to maturity is required before an animal can breed. Thus, the notion that the attainment of a particular body size or composition provides the trigger for initiation of puberty has attracted considerable attention. Examination of the relationship between physical growth and the onset of puberty in primates has been restricted primarily to the human female, with menarche providing the usual endpoint. Attention was first drawn to a close correlation between the age of menarche and the rate of skeletal maturation.[335,336] Later, Frisch and Revelle,[337] stimulated by studies of the relationship between body weight and the onset of puberty in rats,[338] drew attention to their observation of a relationship between menarche and the attainment of a mean body weight of approximately 47 kg. This association led the latter workers[337] to propose the hypothesis "that attainment of a body weight in the critical range causes a change in metabolic rate, which, in turn", is responsible for an increase in hypothalamic drive to the pituitary gonadotrophs.

Shortly after the formulation of this so-called "critical weight hypothesis", Frisch and her colleagues modified their views to include the concept of threshold or critical body composition for puberty.[339] Specifically, menarche was argued to be most closely correlated with attainment of a particular proportion of body fat. According to such hypotheses, the secular trend toward an earlier age at menarche (see previously) is attributed to attainment of a threshold body weight or composition at a progressively younger age as a result of a corresponding secular improvement in the standard of living, including better nutrition and health care. Although the specific hypotheses of Frisch and her colleagues have been criticized on the basis of methodological and statistical grounds,[340–344] the isolation in 1994 of leptin, a protein that is secreted by adipocytes and regulates feeding behavior and body weight by providing the hypothalamus with information on fat mass,[345,346] rekindled interest in the Frisch hypotheses. Indeed, an enormous effort was expended over the ensuing decade to examine the role of this adipocyte hormone in the timing of gonadarche. Next we consider the evidence for a role for leptin and other putative endocrine and metabolic signals that might relay information on somatic development to the brain.

Leptin

Leptin, encoded by the leptin gene (*LEP*), is primarily secreted by adipocytes and regulates feeding behavior and body weight by providing the hypothalamus with information on fat mass and energy status (see Chapter 35). Leptin action is mediated by leptin receptors, which are single transmembrane domain receptors. Six isoforms of the leptin receptor have been described; all represent alternatively spliced products derived from a single gene (*LEPR*) located in man at chromosome 1p31.3.[347] The phenotype of individuals with deficits in leptin signaling, resulting from loss of function mutations in either *LEP* or *LEPR* include morbid obesity, abnormal eating behaviors, and lack of spontaneous pubertal development.[348–350]

Cross-sectional and longitudinal studies of both boys and girls indicate that circulating leptin concentrations increase during pubertal development.[351–355] Moreover, the pubertal increases in circulating leptin concentrations in children are inversely related to changes in levels of the soluble leptin receptor,[356,357] indicating that the rise in bioavailable leptin at this stage of development may be more robust than that of total immunoactive leptin. The binning of leptin data with respect to Tanner stages of pubertal development in the foregoing studies, and the paucity of detailed longitudinal information on both leptin levels and LH pulsatility during transition from Tanner stage 1 to stage 2, however, does not allow the question of whether circulating leptin increases immediately prior to the initiation of gonadarche to be addressed. One exception to this situation has been provided by the longitudinal study of eight boys in whom increments in circulating leptin concentrations over a period of several months to a year were reported to occur prior to nocturnal elevations in testosterone secretion.[358] As previously noted,[359] however, the leptin levels reported in the latter study were extremely variable, and the seemingly tantalizing increments of circulating levels of leptin were actually rather trivial in many of the subjects. Moreover, in contrast to normal puberty, circulating leptin concentrations were unchanged at the time gonadarche was initiated in boys with constitutional delay in growth and puberty.[360]

The relationship between circulating leptin levels and the onset of gonadarche in other species of highly evolved primates has been examined in the rhesus monkey. In the female, circulating leptin levels at the initiation of gonadarche, as identified by increased nocturnal LH secretion, have been reported to be greater than those observed at 14 months of age.[361] In the male monkey, however, the longitudinal relationship between the onset of nocturnal testosterone secretion and plasma leptin levels is unremarkable.[362] Results from cross-sectional studies of the male monkey[363,364] are also consistent with the view that gonadarche in the male is triggered in the absence of a rise in circulating leptin. Moreover, studies of the agonadal male rhesus monkey suggest that both the restraint and the resurgence of GnRH pulse generator activity during infancy and at the end of the juvenile phase of development, respectively, occur in the absence of changes in circulating leptin concentrations.[362,365]

Daily administration of recombinant human leptin in intact female rhesus monkeys, starting at 1 year of age, was associated with increased circulating LH concentrations at 14 months of age, when compared to those in the control group.[361] Nocturnal LH secretion was also detected earlier in the leptin-treated group, and this was associated with a 2-month earlier menarche, although premature menstrual cyclicity was not observed. In agonadal males, however, a continuous 16-day intravenous infusion of recombinant human leptin did not trigger precocious activation of GnRH pulse generator activity.[366]

In contrast to the lack of compelling data to support the hypothesis that increased leptin signaling provides the pivotal cue timing the pubertal resurgence of pulsatile GnRH release, several clinical observations support the idea that this hormone is nevertheless obligatory, in a permissive sense, for initiation of this critical developmental event.[359] Over the last 15 years, leptin replacement has been administered to several patients with leptin deficiency due to *LEP* mutations. When administered at an age appropriate for puberty, leptin promoted pubertal development. Importantly, there has been no evidence of premature puberty in younger children following replacement treatment.[349,367–369] Moreover, among children with GnRH-dependent precocious puberty, leptin concentrations correlated with body mass index and not pubertal status.[370] These findings provide compelling evidence that the action of leptin, albeit obligatory for the onset of puberty, is nevertheless permissive.[371–374] This action of leptin may require only low circulating levels of the adipocyte hormone because normal pubertal development in both male and female subjects with various lipodystrophies has been reported despite low leptin concentrations.[375,376] Similarly, regular menstruation in underweight postpubertal women has also been observed in the face of marked hypoleptinemia.[377]

The neuronal targets for leptin's action in the hypothalamus to permit puberty to unfold and allow GnRH pulsatility to be maintained postpubertally are an area of considerable contemporary interest, but one that is being pursued primarily in non-primate models (see Chapter 35). While it is generally recognized that GnRH neurons do not express *LEPR*, data obtained in rodents and sheep regarding expression of this receptor by KNDy neurons are inconsistent, and the site of action of leptin may be upstream to the GnRH pulse generator.[378] Interestingly, recent studies of transgenic mice[379–381] indicate that GABA neurons expressing leptin receptors may play a role in puberty onset in this rodent. Since GABA plays a critical role in the control of primate puberty, it will therefore be important to examine whether hypothalamic GABA neurons in the monkey express leptin receptors and, if so, their role in controlling gonadarche is established.

Growth Hormone and Insulin-like Growth Factor-1

Growth hormone (GH) plays a major role in the regulation of somatic growth, and, therefore, the finding that puberty is markedly delayed in children with isolated GH deficiency[382] raises the possibility that GH may be involved in the interface between somatic development and the timing of the onset of puberty. Although this is an attractive notion, it has been generally recognized that changes in GH secretion during puberty, at least in man, are the consequence, rather than the cause, of the underlying neurobiological events triggering puberty.[9,383] Thus, the pubertal rise in GH secretion in humans[384–388] and the concomitant increase in circulating insulin-like growth factor-1 (IGF-1) concentrations,[389–392] which has also been reported for the rhesus monkey,[393,394] baboon,[47] and chimpanzee,[26] are absent in agonadal subjects of pubertal age[395] and may be mimicked in sexually infantile children or in subjects with delayed puberty by treatment with sex steroids.[396–399] Moreover, the enhanced GH secretion that is observed in precocious puberty is suppressed when the drive to the pituitary–gonadotrope axis is interrupted with GnRH analog treatment.[391,400]

The notion of a causal relationship between the neuroendocrine axis governing growth and that regulating gonadarche has been championed by Wilson and his colleagues at the Yerkes National Primate Research Center, based primarily on their studies of the female rhesus monkey. This was prompted by the finding that, in the perimenarcheal female rhesus monkey, the onset of the pubertal rise in LH secretion was preceded by about 6 months with a marked daytime rise in the circulating GH concentration.[83] Moreover, the same temporal relationship between the pubertal LH increase and GH secretion was observed in animals that were ovariectomized premenarcheally and subsequently treated with estradiol,[83] suggesting that, in contrast to man, the increase in GH secretion at this stage of development may not be triggered by the rise in estrogen secretion. An increase in GH secretion in association with the pubertal resurgence in pulsatile GnRH release has also been reported for the agonadal male rhesus monkey.[401,402]

The effects of perturbing GH status on the timing and tempo of puberty in the female rhesus monkey have been reported on several occasions. Treatment of premenarcheal monkeys with human GH was associated with a premature increase in pubertal LH secretion, menarche, and first ovulation.[403] On the other hand, IGF-1 administration to premenarcheal female rhesus monkeys advanced only first ovulation,[404] and attempts to suppress GH and IGF-1 in premenarcheal females by treatment with somatostatin delayed only first ovulation.[394,405] Moreover, the timing of the pubertal LH increase in ovariectomized estrogen-treated monkeys was not influenced by GH treatment,[403] and IGF-1 administration to untreated agonadal females was

without effect on this parameter of pubertal development.[243] Taking the foregoing observations together, it seems reasonable to conclude that it is unlikely that GH secretion dictates the timing of the pubertal resurgence in pulsatile GnRH release, but rather it modulates the tempo of pubertal development following activation of the critical neurobiological event triggering increased GnRH release. One mechanism whereby GH accelerates the tempo of pubertal development in the female monkey appears to involve an action of IGF-1 at the hypothalamic and/or pituitary level to impair the negative feedback action of estradiol secreted by the pubertal ovary on gonadotropin secretion[243]; Figure 32.14.

Melatonin

Several lines of indirect evidence have been employed to support the idea that pineal melatonin may provide a signal that is read by the putative somatometer. Circulating concentrations of this indolamine in prepubertal children and female monkeys are higher than those in adults, and a decline in this plasma hormone occurs during juvenile and pubertal development.[406–412] The production rate of melatonin, as reflected by studies of the urinary excretion of 6-hydroxymelatonin, the principal metabolite of this pineal hormone, either declines or remains constant during childhood and puberty.[413–416] It has been known since the beginning of the twentieth century that pineal tumors may be associated with sexual precocity in man,[417] and the argument is made that those pineal tumors that destroy the parenchyma of the gland suppress the release of melatonin[418], which, in turn, leads to a premature reactivation of pulsatile GnRH release. It should be noted, however, that pineal tumors may themselves secrete gonadotropic substances[419,420] and may

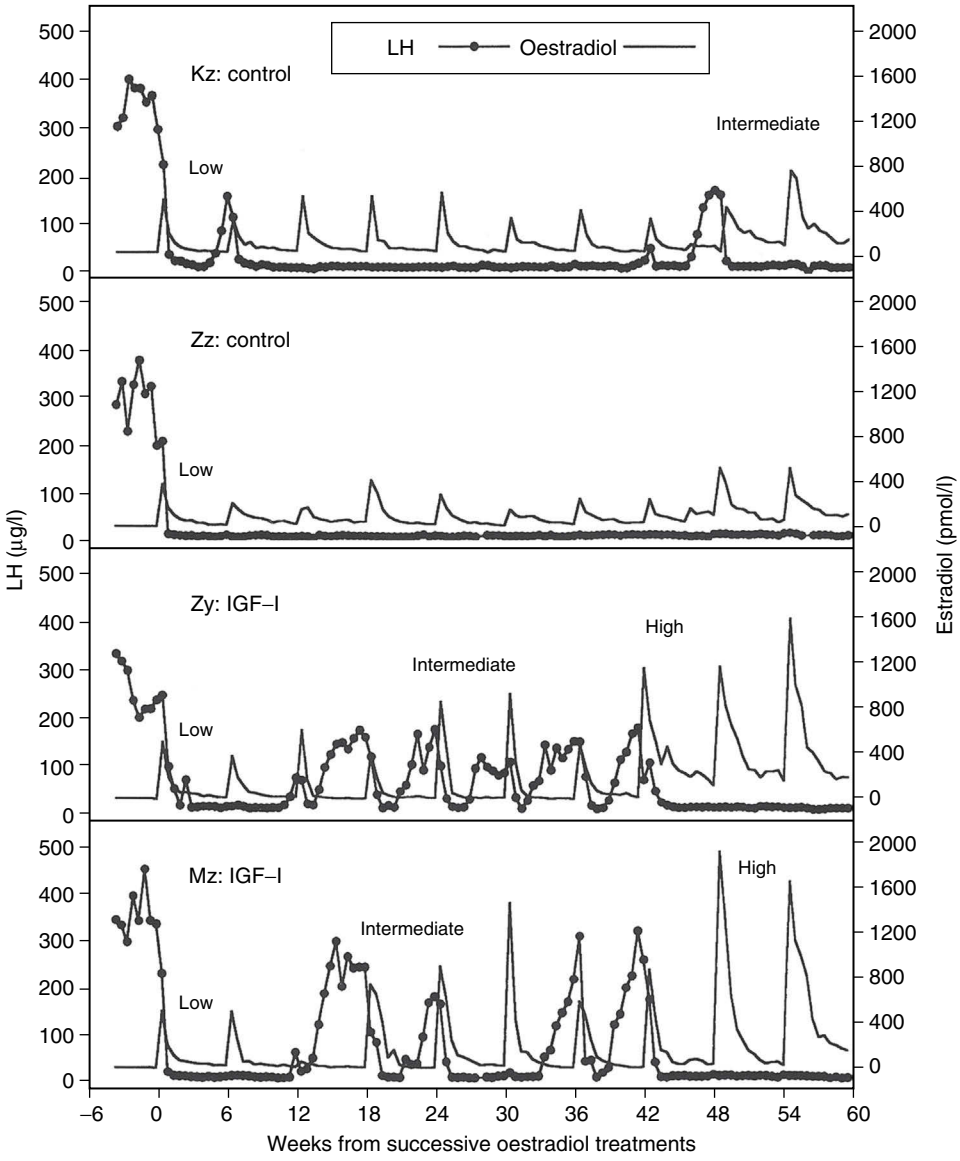

FIGURE 32.14 Developmental changes in serum LH concentrations (solid circles) in four female monkeys ovariectomized at approximately 13 months of age: two served as control (top two panels) and two were treated with IGF-1 (60–300 μg/day) from 16 to 18 months of age (bottom two panels). All four animals were replaced with estradiol starting at approximately 25 months of age (week 0). For this purpose, steroid-containing pellets were implanted subcutaneously every 6 weeks and resulted in "spikes" of circulating estradiol (continuous line) lasting approximately 3 weeks. The doses of estradiol used (low, intermediate, and high) are shown for each monkey. *Reproduced by permission from Ref. 243. © Society for Endocrinology 2005.*

impinge on adjacent areas of the hypothalamus and, thus induce premature sexual development by the same mechanism that leads to other forms of lesion-induced precocious gonadarche (see above). In patients with idiopathic precocious gonadarche, circulating melatonin concentrations are lower than those in age-matched individuals and similar to those of normal pubertal subjects.[421] Moreover, the suppression of circulating melatonin concentrations in patients with this form of precocity is not the consequence of elevated gonadal hormones, since down-regulation of the pituitary–gonadal axis following treatment with GnRH analogs fails to restore melatonin to normal levels.[421,422] The latter result is consistent with the finding that the prepubertal decline in circulating melatonin concentration in the female rhesus monkey is independent of the changes in estradiol secretion at this stage of development.[423]

If elevated circulating melatonin levels during prepubertal development are indeed monitored by a somatometer, which, in turn, holds pulsatile GnRH release in check, then removal of the pineal gland during this phase of development should lead to a premature reactivation of the GnRH pulse generator. Studies in the monkey, however, suggest that this is not the case.[208,424] Pinealectomy in agonadal males during the prepubertal hiatus in pulsatile GnRH release does not appear to influence the timing of the pubertal reactivation of the GnRH pulse generator (Figure 32.15), and the decline in GnRH pulse generator activity during infancy in pinealectomized monkeys is indistinguishable from that in sham-castrated animals.[424] Moreover, increasing the duration of the nocturnal elevation in circulating melatonin in the female rhesus monkey during the third year of life advances the age of menarche and ovulation.[425] This latter finding probably reflects an action of the control system governing seasonal influences on the hypothalamic–pituitary–ovarian axis, rather than a perturbation of the neurobiological brake that dictates the duration of the prepubertal hiatus in pulsatile GnRH release. Thus, it must be concluded that there is no compelling evidence for the view that the signal for the pubertal resurgence of pulsatile GnRH release is provided by a decline in circulating melatonin concentrations.

Metabolic Hormones and Substrates

Steiner and his colleagues have proposed that the reduction in GnRH secretion from infancy to puberty may be the consequence of decreased availability of certain metabolic hormones or substrates.[426,427] These investigators proposed that, in the juvenile primate, the GnRH pulse generator is subjected to a metabolic milieu similar to that associated with fasting states in adults, such as in anorexia nervosa, in which hypothalamic GnRH release also appears to be compromised.[428,429] In a somewhat similar vein, Winterer et al.[430] have proposed that recognition of a critical level of available calories by the primate brain leads to the reawakening of the dormant GnRH pulse generator and, therefore, to the initiation of gonadarche. According to the latter hypothesis, a reduction in available calories below the critical level in adults could lead to impairment in GnRH release.

Several lines of circumstantial evidence, which have been reviewed by others,[426,427,430] may be used to support such metabolic theories for the onset of puberty. The inhibition of gonadotropin secretion in adult monkeys and human beings that result from restricted food intake may be observed, as in the case of the prepubertal hiatus in LH and FSH release, in the absence of gonadal steroids.[431,432] Moreover, the pattern of hypothalamic GnRH pulse generator activity, as reflected by pulsatile patterns of LH secretion in female anorectics, appears to resemble closely those described for prepubertal and pubertal girls.[428] Anorexia nervosa and its related disorder,

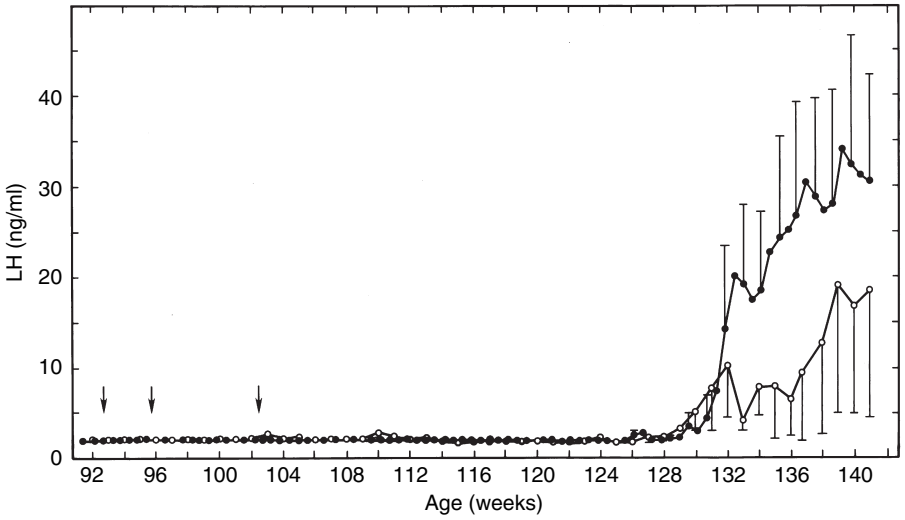

FIGURE 32.15 Failure of pinealectomy to induce a precocious pubertal reactivation of the GnRH pulse generator (as reflected by circulating LH concentrations) in agonadal male rhesus monkeys. Data for pinealectomized animals (•-•, $N=3$) are compared to those from a similar group of pineal-intact monkeys (O–O, $N=3$). *Arrows* indicate the age at pinealectomy. The time courses of FSH secretion in the two groups of animals (not shown) were similar to that of LH. *Redrawn with permission from Ref. 208.*

bulimia nervosa, however, are complex diseases characterized by psychosocial features, including difficulties with emotional regulation and adult autonomy, and tendencies toward obsessive-compulsive personality disorder in addition to weight loss. Moreover, dysregulation of the hypothalamic–pituitary adrenal axis, characterized by elevated cortisol concentrations in the face of normal ACTH concentrations, reduced peripheral metabolism of cortisol, and lack of suppressibility of ACTH by dexamethasone, is common.[433] Overactivity of the serotonergic system is also present.[434] Although no specific genes have been identified, family and twin studies support the hypothesis that genetic factors influence the risk for both these disorders.[435] Thus, anorexia nervosa represents the outcome of complex interaction between multiple genetic factors, early life experiences, and environment during the peripubertal years.

Although many of the metabolic sequelae of short-term fasting (such as the decline in circulating levels of glucose, amino acids, and insulin, as well as the increase in plasma concentrations of free fatty acids and β-hydroxybutyrate) tend to be more pronounced in juveniles than adult primates,[436–438] these metabolic adaptions do not, in general, appear to emerge until 12 h or more into a fast. Thus, it is difficult to envisage how such a nutritional signal would be relevant to pubertal timing in well-nourished monkeys or children.

With the exception of Steiner and his colleagues, few investigators have attempted to address directly the foregoing metabolic hypotheses of puberty. In a technically difficult study, the latter workers examined the gonadotropin response to a sustained intravenous infusion of glucose and amino acids administered to castrated male crab-eating monkeys between 18.5 and 31 months of age.[426,427] This hyperalimentation, which resulted in a 75% increase in daily caloric intake and, presumably, stimulated insulin secretion, was associated with an elevation in circulating LH concentrations in approximately 50% of the animals studied. In evaluating this tantalizing observation, it should be noted that in the closely related rhesus macaque, gonadotropin secretion in prepubertally castrated males may be first detected as early as 2 years of age.[49] It is, therefore, not inconceivable that spontaneous reawakening of the hypothalamic GnRH pulse generator may have been initiated during hyperalimentation in the older crab-eating macaques. The latter possibility would be excluded, of course, by a return of circulating LH to preinfusion control levels following termination of hyperalimentation, but such data were reported for only one animal. Unfortunately, these observations made 30 years ago have not been pursued.

It has long been recognized that insulin resistance increases during puberty in children,[9,439–441] although it has generally been considered that this phenomenon, like the pubertal elevation in GH, is a consequence of gonadarche. A recent longitudinal study of a modest number of children, however, suggests that the increase in insulin resistance may be a prepubertal event.[442] The notion that insulin may serve as a metabolic trigger for puberty is reinforced by the proposal that, in the context of the regulation of appetite, the primate hypothalamus has the capacity to monitor circulating insulin levels.[443,444] Further credibility would be given to the foregoing speculation by the demonstration that fasting insulin levels also rise in agonadal children and monkeys concomitantly with the reawakening of the GnRH pulse generator.

Ghrelin is a small peptide secreted predominantly by the stomach, and resistin and adiponectin are two recently described hormones secreted by adipocytes[445–450]: all three factors play a role in nutritional/metabolic homeostasis, but a role in the onset of gonadarche or adrenarche has not been established.

Other Factors

Although adrenarche typically precedes puberty,[3] the possibility that, in man, a putative somatometer responds either directly or indirectly to maturation of the adrenal appears to be very unlikely. The most compelling evidence that may be cited for the latter view is the finding that the onset of gonadarche does not appear to be noticeably delayed in children with primary adrenal insufficiency present before the age of adrenarche.[451] Conversely, premature adrenarche is not associated with any apparent signs of precocious gonadarche,[451] and idiopathic GnRH-dependent precocious gonadarche is typically observed prior to adrenarche.[452] From a comparative perspective, it is also difficult to argue that a rise in adrenal androgen secretion is a critical event in the pubertal reawakening of the hypothalamic GnRH pulse generator in other primates, since adrenarche, when noted in Old World monkeys (rhesus macaque), was observed to occur during infancy[3,6]; also see above. Moreover, adrenalectomy during the first week of life in agonadal male rhesus monkeys did not delay the timing of the reactivation of the GnRH pulse generator (Figure 32.16). As with many other factors, the prepubertal status of circulating adrenal androgen levels have been reported to be correlated to pubertal timing in children and therefore these steroids may modulate the precise timing of the reactivation of pulsatile GnRH release at the termination of juvenile development.[453,454]

Exposure to supraphysiological concentrations of plasma androgens during juvenile development may lead to premature reawakening of the hypothalamic GnRH pulse generator. Children with congenital adrenal hyperplasia (CAH) and advanced skeletal maturation may exhibit signs of GnRH-dependent precocious puberty (e.g., sleep-augmented LH release[455]) or, more frequently, may progress into a state of true sexual maturity despite

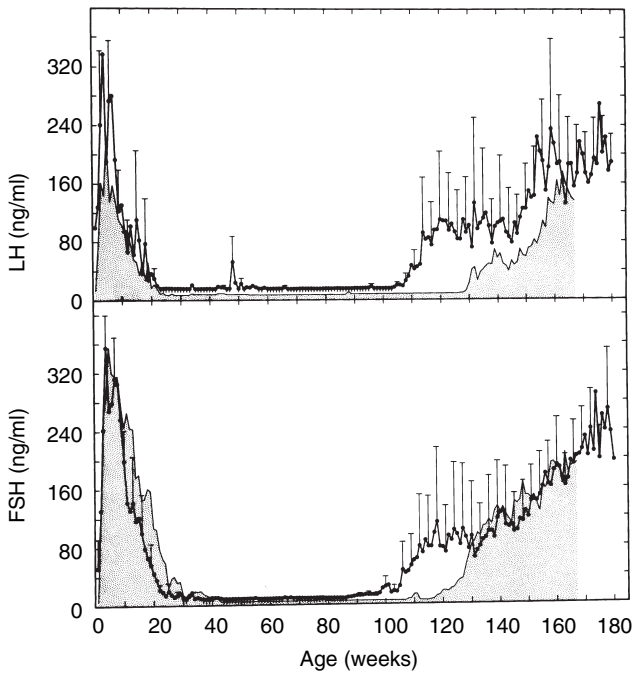

FIGURE 32.16 Time courses of circulating mean LH (top panel) and FSH (bottom panel) concentrations from birth until 166–180 weeks of age in a group of rhesus monkeys orchidectomized at 1 week of age (*stippled area*, $N=4$) and in a group of similar agonadal males adrenalectomized at 4 weeks of age (•: $N=3$, birth to 40 weeks; $N=5$, 41–180 weeks). The apparent earlier initiation of the pubertal mode of open-loop gonadotropin secretion in adrenalectomized animals is attributable to one animal. In the remaining four adrenalectomized animals, the pubertal rise in FSH secretion was not observed until 136 weeks of age or later. Vertical lines above data points indicate SEMs. *The data for castrated males are redrawn with permission from Ref. 49, and those for castrated/adrenalectomized males are, in part, redrawn with permission from Ref. 252.*

appropriate corticosteroid replacement treatment.[451,456,457] Interestingly, long-term testosterone treatment of rhesus monkeys during the first year of life mimics the effects of adrenal hyperplasia in man. In the female monkey, Van Wagenen[458] found that the age of menarche was advanced by 1 year, and in the agonadal male monkey, one of the authors' laboratories reported that early testosterone treatment led to a remarkably precocious pubertal increase in LH secretion.[459] The mechanisms whereby supraphysiological and early exposure of androgens appear to result in the premature reawakening of the hypothalamic GnRH pulse generator are not known. Since treatment of agonadal male monkeys during the first year of life with high levels of estradiol or dihydrotestosterone did not accelerate the onset of the pubertal resurgence in LH release[459], it seems reasonable to postulate that the effect of testosterone may be mediated by high levels of estradiol produced by local aromatization within the specific hypothalamic areas. Alternatively, the influence of excess androgen on the timing of the pubertal process may be mediated indirectly by a metabolic signal induced by steroid action on peripheral structures.[330] In this regard, it is becoming increasingly apparent that bone serves as an endocrine organ subject to the influences of sex steroids and, in males, the osteoblast-derived hormone osteocalcin promotes testosterone biosynthesis.[460] Whether osteocalcin influences the central component of the hypothalamic–pituitary–gonadal axis has not been examined. In any event, the appeal of the notion that attainment, by whatever mechanism, of a critical level of somatic maturation provides a trigger for the initiation of puberty is again reinforced.

In summary, the notion of a growth-tracking device in the brain that monitors body size and synchronizes the attainment of a degree of somatic development sufficient to bear and nurture offspring with the pubertal reactivation of GnRH pulsatility and therefore the onset of reproductive competence continues to be an attractive proposal. However, at the time of writing, there are no compelling candidates for the circulating signal that is read by the putative somatometer, and until such cues are identified the idea will remain hypothetical.

Pubertal Clock

Only scant attention has been paid to the possibility that the timing of the onset of puberty is governed by an endogenous biological clock. Although there is no doubt that the mammalian CNS is endowed with timekeeping systems capable of tracking both circadian and circannual periods (see Chapters 26 and 34), the role, if any, of such mechanisms in dictating the ontogeny of the primate hypothalamic GnRH pulse generator has not been established. It is to be expected, however, that if such endogenous clocks are involved in the regulation of hypothalamic GnRH secretion throughout development, then selective damage to the timing mechanism would lead to a major alteration in the age at which puberty is initiated. In this regard, it is generally recognized that important timekeeping functions of the mammalian brain are subserved by the suprachiasmatic area of the hypothalamus.[461] As noted earlier, attempts to dissociate the hypothalamic GnRH pulse generator in the prepubertal female rhesus monkey from the influence of more rostral brain regions, including the suprachiasmatic nucleus, by surgically transecting neural pathways between these two areas have not consistently resulted in a major alteration in the timing of menarche and other indices of puberty.[331,332] However, this surgical intervention in juvenile animals failed to abolish the diurnal rhythm in cortisol secretion.

Placement of lesions in the anterior hypothalamus of premenarcheal female rhesus monkeys has also been reported to be without effect on the age of menarche.[462] In a subsequent study of the same problem, however, a significant number of prepubertal female monkeys with

anterior hypothalamic lesions failed to thrive, and, when data from these animals are excluded from numerical analysis, a lesion-induced advancement in menarche and first ovulation emerges.[324] In humans, on the other hand, lesions of the anterior hypothalamus have generally been observed in association with delayed sexual maturation or hypogonadism.[463] Moreover, the specificity of the neurosurgical procedure employed for the placement of anterior lesions in the monkey is unclear, because similar lesions in the posterior hypothalamus also advance menarche and first ovulation in intact animals and the pubertal reinitiation of open-loop LH secretion in ovariectomized monkeys.[324] The latter finding is consistent with the generally accepted view that posterior hypothalamic lesions that involve the tuber cinereum and mammillary bodies result in precocious puberty in humans.[9,464,465] As discussed previously, the puberty-advancing action of some lesions may result from increased expression in the vicinity of the lesion of glial-derived growth factors, such as TGFα and neuroregulins. Although further study of the sequelae of destructive hypothalamic lesions in the primate brain should not necessarily be discouraged, such an approach to date has not provided significant insight into the nature of the control system that times the pubertal reawakening of the hypothalamic GnRH pulse generator.

GENETICS, EPIGENETICS AND PUBERTY GENES

As with other complex developmental processes, multiple genes are likely to contribute to the timing and tempo of puberty, and some of these may be coassociated with parameters of pubertal growth velocity and others not.[100,466,467] In this regard, the recognition that genetics play a role in regulating the timing of gonadarche has been highlighted.[468] Greater concordance in age of menarche and other indicators of the progression of gonadarche, such as peak height velocity and plasma LH concentrations, have generally been found in monozygotic twins when compared to dizygotic twins.[469–476] The impact of genetics on the timing of gonadarche is further indicated by the findings of a high degree of heritability in the age of menarche[473,475] and, in the United States, of a significantly earlier age of menarche in Afro-American girls than in their white counterparts.[119,477–479] Similarly, girls in London of Afro-Caribbean and Indo-Pakistani parentage have an earlier age of menarche than white girls of British parentage.[480] In addition, precocious gonadarche in girls may be familial and appears to be transmitted in an autosomal dominant mode.[481]

We have earlier suggested that the term "puberty" gene should be restricted to those genes that are specifically involved in the timing of either gonadarche or adrenarche.[3] The most likely, although theoretically not only, site for expression of "puberty" genes would be the brain, and probably the hypothalamus. In the case of gonadarche, the proteins encoded by these genes would determine the age of the pubertal resurgence of pulsatile GnRH release by regulating the timing of the application or withdrawal of transynaptic and/or glial inputs to the GnRH pulse generator of the juvenile hypothalamus. Conceivably, such putative "puberty" genes could time the resurgence of pulsatile GnRH release not only by triggering a hypothalamic signal at puberty but also by determining the duration of the prepubertal brake on pulsatile GnRH release or by timing the "turn off" of the GnRH pulse generator during infancy.

The foregoing definition of a puberty gene would eliminate *KISS1* and possible *TAC3*, which, as discussed previously, are currently viewed to be GnRH pulse-generating genes. Similarly, mutations of many genes involved in the differentiation and early development of the hypothalamic–pituitary–gonadal axis have been reported.[482] Mutations in these genes, which include *FGFR1*, *FGF8*, *KAL1*, *PROK2*, *PROKR2*, *CHD7*, *NELF*, and others, have profound effects on pubertal development. However, as previously argued,[483] such mutations have not clarified our understanding of the control system responsible for the timing of gonadarche. For example, mutations of the X-linked *KAL-1* gene, which encodes anosmin, an extracellular glycoprotein that is necessary for the early embryonic migration of GnRH neurons into the hypothalamus,[484] are associated with hypogonadotropic hypogonadism and absence of puberty. While such mutations have provided significant insight into the fetal development of the reproductive axis, they have contributed little to our understanding of the control system that governs the timing of the pubertal resurgence of pulsatile GnRH release.

Recently, genome-wide association studies indicate that several specific loci on the human genome are associated with variations in the age at menarche (AAM).[485–489] A locus with a high frequency of single nucleotide polymorphisms (SNPs) that was consistently and strongly associated with an earlier AAM was found on chromosome 6 (6q21) in the region of *lin-28 homolog B (Caenorhabditis elegans) LIN28B*: a gene that encodes for a micro-RNA binding protein.[490] The differences in AAM in subjects with and without variants in the region of *LIN28B* are relatively small (1–2 months) compared to the recognized range for the AAM in the population at large that may extend from 10 to 16 years of age.[3] In one of the foregoing studies, an association between the *LIN28B* locus and advanced voice breaking and/or advanced pubic hair stage was also reported for boys.[486] It should be noted, however, that when an association between either a genetic locus or a specific gene and the timing of puberty is established, it remains to

be determined whether the relationship is causal, and if so whether the genetic parameter is directly determining the timing of the pubertal resurgence of GnRH release, or simply influencing permissive factors such as those that reflect metabolic and growth status, and that are required for this developmental event to be triggered.

Another contemporary and powerful genomics technique that has recently been applied to examine the genetic basis of puberty onset is whole exome sequencing. Using this methodology, the entire coding region of the genome of members of families with a particular disorder of pubertal development may be sequenced and compared to a "normal" genome. Using this approach, central precocious puberty in seven girls and five boys from 12 families has been found to be associated with mutations in the gene encoding for makorin RING finger protein 3 (*MKRN3*).[491] All affected individuals inherited the disorder-associated allele from their fathers, indicating that only the paternal allele is expressed.[491]These mutations are predicted to result in loss of function of the protein. While the function of MKRN3 is not fully understood,[492] the makorin family of proteins contains a particular zinc-finger motif that has been associated with ubiquitination, a process that is involved in protein trafficking, which in some cases leads to protein degradation. Puberty in these individuals was observed as early as 5–6 years of age in both sexes, and therefore *MKRN3* may well be a puberty gene. Clearly, examination of expression of this gene in the monkey hypothalamus throughout postnatal development is likely to be very informative.

A third novel approach to explore the genetic basis underlying the timing of puberty is to first describe global changes in hypothalamic gene expression using either microarrays or RNAseq across peripubertal development; then, computational (in silico) biology is applied to examine whether the genes that are empirically determined to change with pubertal development are likely to be members of networks of functionally linked hypothalamic genes.[493,494] To date, this approach, which has been pioneered by Ojeda and his colleagues, has been applied only to non-human primate models. In this regard, it is proposed that developmental changes in a network of genes encoding for cohorts of transcription factors, such as tumor suppressor genes, lie—in a molecular sense—upstream of the GnRH pulse generator.[493] It is further argued that such gene networks serve as a governing hierarchy to orchestrate the increase in pubertal GnRH release and therefore the timing of puberty. However, until information is forthcoming on the phenotype and location of the neurons/glial cells in which the puberty associated genes are expressed, it will be difficult to integrate such in silico hypotheses into our thinking of puberty that has evolved from more classical approaches. Nevertheless, the potential significance of this approach is indicated by the finding that one of these transcriptional regulators, enhanced at puberty 1 (EAP1), is expressed in kisspeptin neurons in the arcuate nucleus of the monkey, and its expression in the hypothalamus increases at the time of puberty in the female.[495,496] Moreover, knockdown of *EAP1* using a lentivirus approach interrupts menstrual cyclicity in the female monkey,[497] and an SNP upstream of the *EAP1* gene has been associated with irregular menses in this primate.[498]

The recent finding that global inhibition of DNA methylation by intraperitoneal injections of 5-azacytidine in prepubertal rats resulted in the delay of vaginal opening, indicating that epigenetic regulation of gene expression may be important for timing puberty, has led to yet another strategy for interrogating the genetic basis of this developmental process,[499] i.e., to search for hypothalamic genes that are epigenetically regulated during peripubertal development. In this regard, studies of the rat have led to the identification of a cohort of genes that encode for either a group of transcriptional "silencer" proteins known as the polycomb group or proteins with which the polycomb group interact.[499] In the pubertal rat, expression of these polycomb genes is downregulated by DNA methylation leading to a reduction in the silencer proteins they encode. Interestingly, two genes of the polycomb group were found to be expressed in neurons of the rat arcuate nucleus (some of which coexpressed *Kiss1*), and overexpression of one of these genes in neurons of this nucleus, which was targeted by a lentivirus construct, resulted in a decrease in *Kiss1* expression in association with delayed vaginal opening and a disruption of GnRH pulsatility in MBH explants.[499]

As argued previously, we consider that *KISS1* is a GnRH pulse-generating gene and not a puberty gene.[168] Moreover, GnRH pulse generator activity is robust during infancy in monkeys and man, and in these species puberty is triggered by a reactivation of this neurobiological mechanism after a prolonged period of relative dormancy during juvenile development. It would therefore be of great interest to establish whether epigenetic silencing is involved in the "turn off" of pulsatile GnRH release during infancy in the monkey. While the specific question of whether postnatal epigenetic changes in polycomb genes occur in the primate hypothalamus remains to be addressed, one of the authors' laboratories has recently found that DNA demethylation of the *GnRH* gene in the MBH occurs across puberty in male rhesus monkeys in association with a pubertal increase in GnRH mRNA.[268] Therefore, epigenetic regulation of hypothalamic gene expression is likely to be a contributing factor for the mechanism of puberty onset.

It is highly likely that the number of puberty genes that are expressed in the primate hypothalamus will expand dramatically over the next few years as high throughput

approaches to genomics are increasingly applied to investigate this developmental process. Whether these new anticipated data will solve the fundamental nature of the physiological control system timing the onset of primate puberty—i.e., whether the pubertal reactivation of the GnRH pulse generator activity and therefore the onset of gonadarche in primates is timed by a pubertal clock or a somatometer is less clear.

FETAL PROGRAMMING AND THE ONSET OF PUBERTY

Epidemiological observations and subsequent clinical and experimental data have demonstrated an association between intra-uterine growth retardation and increased risk for several endocrine and non-endocrine disorders in adulthood.[500] In the case of pubertal development, adrenarche has been reported to be precocious and/or exaggerated in both boys and girls born small for gestational age (SGA), particularly those who show accelerated weight gain during early childhood. In contrast to adrenarche, the timing of menarche in girls born SGA does not appear to be markedly different from girls born at an appropriate size for gestational age.[501–503] In boys born SGA the timing of puberty appears to be normal, although sub-fertility has been reported postpubertally.[503,504]

The role of fetal androgen in programming the neural component of the hypothalamic–pituitary–gonadal axis, and therefore potentially in timing the onset of gonadarche, merits consideration. Testosterone secretion by the fetal testis is presumably responsible for the greater frequency of pulsatile GnRH release in infant male monkeys compared to that in age-matched females (Figure 32.2), and for the subsequent dimorphism in the developmental pattern of GnRH release as reflected by gonadotropin levels in agonadal monkeys and humans (Figures 32.4 and 32.5). Prenatal exposure of female monkeys to elevated levels of androgens administered in large quantities to their mothers has been associated with increased LH pulse frequency, post-natal hyperandrogenism, insulin resistance, and decreased pancreatic beta cell function.[505] These data suggest that the prenatal androgen milieu may influence the risk to develop polycystic ovary syndrome. Additionally, androgen exposure early during gestation in the monkey has been reported to impair oocyte developmental competence.[506] Girls with classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency are exposed to elevated levels of androgens during fetal development. Despite appropriate glucocorticoid replacement therapy, women with CAH can develop ovarian hyperandrogenism, irregular menses, and infertility.[507]

EXTEROCEPTIVE CUES AND THE TIMING OF PUBERTY

Season

Reproduction in many species of primate involves a seasonal pattern in breeding,[508] which is presumably dictated, as in nonprimate species, by photoperiod and possibly other environmental cues (see Chapter 34). The rhesus monkey living outdoors provides a striking example of a seasonal breeding primate: ovulation generally occurs during the fall, and births are usually restricted to the spring.[509] Thus, as previously noted by Rowell[97] and graphically demonstrated by Wilson et al.[393] and Terasawa et al.,[82] the typical feral female experiences her first and second breeding season as an infant and juvenile of approximately 6 and 18 months of age, respectively; menarche occurs during the third breeding season, at approximately 30 months of age, and puberty is completed with first ovulation and conception by approximately 42 months of age during the fourth breeding season. Spring-born females that exhibit unusually early or late first ovulation do so 1 year before or after the age at which ovulation typically occurs.[393] Moreover, when females that are born in the fall, because of contrived laboratory conditions, are reared from birth in an outdoor environment, first ovulation and conception typically occur in the fall at the end of the third year of life rather than at 3.5 years of age as in spring-born animals.[393] Thus, under natural conditions, season imposes a quantum effect on the timing of puberty in the rhesus monkey, and, as previously remarked by Rowell,[97] the onset of menarche or first ovulation is better predicted by counting time in breeding seasons than in months. An analogous quantal relationship between breeding season and the initiation of puberty has also been reported for male rhesus and bonnet macaques[45,510] and for male squirrel monkeys.[46]

That the hypothalamic GnRH pulse generator is an important relay station in mediating the influence of season on the timing of puberty is indicated by the finding that testicular quiescence during the nonbreeding season may be reversed in the monkey by intermittent stimulation of the pituitary–gonadal axis with exogenous GnRH.[511] The finding that menarche and first ovulation are advanced in spring-born female monkeys by imposing a short-day pattern of circulating melatonin during the long days of the summer preceding the third breeding season[425] suggests that the pineal gland is an important component of the interoceptive mechanism mediating the seasonal influence on the timing of the onset of puberty in this species as in other seasonal breeders (see Chapter 34).

Although the foregoing discussion underlines the robust effect seasonally related exteroceptive cues

may have on the timing of the onset of puberty in non-human primates, it is important to remember that there is no evidence to indicate that such cues actually regulate the fundamental control system governing the onset of puberty, namely, the neurobiological brake that switches the GnRH pulse generator off in infancy and on at the termination of prepubertal development. It appears that irrespective of season, the latter control is independently programmed, and the capacity for puberty is achieved independently of season. However, in a seasonal breeding species, puberty may be manifest only when the capacity for puberty and the breeding season coincide.

Social Factors

In nonprimate species, it is well established that social cues influence the timing of the onset of puberty.[512] Cognate mechanisms appear extant in certain species of primates, and this is exemplified by the South American marmosets and tamarins. These small equatorial primates live in family groups consisting of a monogamous pair of adults and their offspring.[105] Interestingly, the breeding female appears to suppress the ability of her adult daughters to reproduce,[105,513] possibly by inhibiting ovulation.[514] In Old World monkeys, socially dominant juveniles of both sexes reach puberty earlier than their subordinate peers.[515–518] The neuroendocrine mechanisms responsible for this intriguing relationship between social dominance and the neuroendocrine axis governing reproductive function in macaques is probably related to elevated "social" stress associated with subordinate status.[518] Furthermore, it is to be anticipated that stress impairment of hypothalamic GnRH pulse generator activity is involved in the delay of puberty in subordinate individuals (see Chapter 36). In man, social subordinance prior to puberty also represents one of several "pediatric" stresses (see Ref. 519 for a comprehensive review of this subject), and the role of psychosocial or socioemotional stressors generated by family dynamics and impacting upon children, particularly girls, in timing the onset and tempo of puberty is an area of sociology receiving increasing attention.[519,520] Interestingly, in this regard, some presumed socioemotional stressors, for example, father absence and insecurity as an infant, appear to be associated with an acceleration in the timing of puberty.[519,521–523] The reasons for the opposite effect of "stress" prior to puberty on the timing of this developmental event in Old World monkeys on the one hand and in boys and girls on the other are unclear but have been posited to be related to differences in diet.[518,524] Specifically, children raised under conditions of high stress may consume foods high in fat and sugars.[525]

Another interesting association between behavior and the onset of human puberty first reported in 1981 is the precocious gonadarche that has been reported in children, particularly girls, adopted by European parents from developing world regions such as Asia and South America.[120] Ethnic background does not appear to be involved, and a similar phenomenon has also been observed in immigrant girls arriving with their parents.[120] The mechanisms responsible for the association between adoption and/or migration and puberty in girls remain unclear.

Food Availability

In addition to the impact of food availability during prepubertal development on somatic growth, and therefore the signals posited to be tracked by the putative somatometer (see above), the balance between energy intake and energy expenditure is a critical environmental factor determining whether puberty will progress once growth has reached a pubertal threshold.[110,519,526] In girls menarche is delayed in malnourished individuals,[111,527] while obesity is associated with early breast development and menarche,[528] although this phenotypic prematurity may not be directly related to GnRH pulse generator activity.[529] On the other hand, the increased metabolic demand that may be imposed by strenuous exercise such as long-distance running, gymnastics, and ballet is often associated with delayed or interrupted pubertal development.[530,531] In such individuals, in whom deviation from ideal body weight may be minimal, the frequency of pulsatile LH secretion is less than that in sedentary women during the follicular phase of the menstrual cycle.[532–535] Male wrestlers lose body weight, fat mass, and lean body mass during the competitive season and disruption of the neuroendocrine axes governing testicular function and growth may be observed at this time.[536] These changes reverse within months following conclusion of the wrestling season. Together these findings suggest that metabolic signals, at least those associated with restricted food intake and/or increased exercise, have the capacity to impair the pubertal activity of the hypothalamic GnRH pulse generator. While the impact of undernutrition on gonadarche in boys has received less attention, there is no reason to suspect that the male axis is unaffected in this regard.

The effects of food availability/nutrition on the timing of puberty in non-human primates have received only scant attention. In the female rhesus monkey, high calorie diets were associated with a precocious increase in nipple volume, sex-skin swelling, and menarche in association with increased concentrations of leptin and IGF-1 concentrations, and an increased BMI.[123]

Chronic disease is often associated with inadequate nutrition (and in all probability with stress) leading to impaired GnRH secretion and delayed or interrupted puberty. Children with sickle cell anemia, perinatally acquired HIV infection, diabetes mellitus, chronic renal disease, and inflammatory bowel disease tend to have delayed puberty.[537–539] Among girls with cystic fibrosis, age at menarche was delayed, but correlated with nutritional status and maternal age at menarche.[540] Effective treatment of the underlying disorder is generally associated with restored pubertal progression. For example, following successful renal transplantation, children with chronic renal disease manifest resumption of gonadotropin secretion and progressive pubertal development.

Restricted food intake, imposed experimentally in the post-pubertal rhesus monkey or voluntarily in human subjects with anorexia nervosa, appears to inhibit gonadotropin secretion primarily by an action at a suprapituitary level that is mediated by suppression of the hypothalamic GnRH pulse generator.[432,541] Thus, in the undernourished juvenile, the same inhibitory mechanisms that arrest the hypothalamic GnRH pulse generator in food-restricted adults are presumably activated, and therefore any waning of the neurobiological brake on intermittent GnRH secretion in the juvenile would not become manifest because of the additional and independent check imposed by restricted food intake. Thus, gonadarche—the consequence of the reawakening of the GnRH pulse generator—is further delayed.

In summary, it is reasonable to conclude that those exteroceptive cues associated with season, social status, and nutrition that impact the reproductive axis of adults are unlikely to be affecting the fundamental neuroendocrine changes driving puberty, just suppressing their expression.

DISORDERS OF PUBERTAL DEVELOPMENT

To date, with the exception of one report in the chimpanzee,[542] disorders of puberty in primates have been described only for man, and these have been recently reviewed in detail.[3] Here, we restrict our discussion to those particular disorders that, when their etiology is fully established, are most likely to add major advances to the understanding of the physiology of primate puberty. Also, certain disorders of human puberty that have provided major insight into key signaling pathways (e.g., kisspeptin-KISS1R) regulating pulsatile GnRH secretion have already been discussed earlier and will not be considered in detail in this section. Since the key events in regulating the onset of puberty are the switches that first turn off the GnRH pulse generator in infancy and subsequently reactivate this neural component to initiate gonadarche at the end of juvenile development (see above), examination of the underlying causes of GnRH-dependent disorders of human puberty will likely be the most revealing. Indeed, discovery of novel genes associated with such conditions provides an ever-expanding appreciation of the complexity of the neurobiology of this critical developmental event. This is not to say that GnRH-independent disorders of pubertal development are neither of physiological interest nor of clinical importance. In fact, disorders of gonadal and adrenal steroidogenesis, or the failure of the pituitary gland to undergo normal development, may have profound impacts on growth and sexual development.

Disorders of human pubertal development fall into the broad categories of precocious and delayed puberty, and both may be either familial or sporadic. In the latter, patterns of inheritance include autosomal recessive, autosomal dominant, and X-linked recessive. For affected patients and their families, molecular genetic diagnosis improves identification of asymptomatic affected family members and enables prediction of associated features.

Precocious Puberty

Precocious puberty may be classified as being either GnRH-dependent or GnRH-independent. GnRH-dependent precocious puberty, or more accurately precocious gonadarche, is the result of a premature resurgence or incomplete suppression of the hypothalamic GnRH pulse generator. GnRH-independent forms represent a group of disorders associated with increased gonadal or adrenal sex steroid secretion that occurs in the absence of GnRH-stimulated gonadotropin secretion.

In GnRH-dependent precocious gonadarche, the normal pubertal sequence of hypothalamic events appears to be recapitulated, but the process starts at an inappropriately early age. This disorder is diagnosed more often in girls than in boys, perhaps because suppression of the GnRH pulse generator during childhood and juvenile development in girls is less robust than in boys (see above). While often considered to be idiopathic in girls, an organic etiology can usually be identified in boys. Despite the relative mature physical appearance of children with precocity, their cognitive and emotional development is normal for chronologic age. Thus, the guilelessness and naïveté of such children exposes them to an increased risk of sexual abuse, with affected girls at risk of becoming pregnant.

Hypothalamic hamartomas, congenital malformations comprised of heterotrophic neural tissue usually located on the floor of the third ventricle or attached to the tuber cinereum, are a common etiology of precocious gonadarche.[9] The tumors can be classified as parahypothalamic when attached or suspended from the floor of the third

ventricle, or as intrahypothalamic, in which the mass is enveloped by the hypothalamus and distorts the third ventricle. Hamartomas do not grow over time and do not metastasize. Although gelastic or laughing seizures can be associated with precocious puberty due to hypothalamic hamartomas, the majority of patients do not exhibit neurologic symptoms. Interestingly, histological examination of hypothalamic hamartoma tissue has shown immunoactive GnRH perikarya in association with capillary networks[543–546] and/or astroglial-derived growth factors such as TGFα.[547] The surgical removal of such small peduncled hamartomas in infants with precocious puberty has been reported to result in a complete arrest of sexual development.[543–546] The intriguing possibility that such hamartomas contain an ancillary GnRH pulse generator that is emancipated from normal developmental controls must therefore be considered. On the other hand, astroglial factors such as TGFα may provide an ectopic drive to GnRH neurons with a normal distribution in the hypothalamus.

Other CNS lesions, hydrocephalus, meningomyelocele and certain neurodevelopmental disorders, previous head trauma, CNS infections, CNS granulomatous disease, and CNS radiation may also be associated with GnRH-dependent precocious gonadarche.[548,549] CNS radiation used to treat intracranial tumors or used prophylactically for malignancies can induce precocious GnRH-dependent gonadarche, perhaps as a result of an astroglial response with increased TGFα production.[325]

Precocious GnRH-dependent gonadarche may also be observed in children with virilizing disorders such as CAH and familial male-limited precocious puberty (testotoxicosis) in whom skeletal maturation is significantly advanced. This particular pathophysiological condition can be replicated experimentally in the monkey by exogenous testosterone administration.[458,459] Whether the action of testosterone to prematurely reawaken the GnRH pulse generator is exerted on the CNS or on peripheral tissue such as, for example, the skeleton, is unclear.

The treatment of choice for children with GnRH-dependent precocious gonadarche is a GnRH receptor (GnRH-R) agonist.[549] GnRH-R agonists are modifications of GnRH having greater resistance to degradation and increased affinity for the GnRH-R than the native decapeptide. They are, therefore, perceived by the pituitary as a continuous GnRH stimulation, which induces desensitization of GnRH-R function and leads to decreased gonadotropin secretion. GnRH-R agonists are available as daily injections or depot forms, the latter including leuprolide acetate and histrelin.[550] The major goals of treatment are to prevent further pubertal progression until appropriate for chronological age, and to attain normal adult height. In situations of coexisting GH deficiency, combined treatment with recombinant human GH may be helpful to preserve height potential.

Delayed Puberty

In the context of understanding the timing of normal puberty, constitutional delay of growth and puberty is of most interest, because this condition, which is currently considered an extreme of the normal variation of pubertal timing, aggregates in families and therefore must have a genetic basis (see above). Typically, gonadarche, adrenarche, and skeletal maturation are delayed. Constitutional delay is a diagnosis of exclusion because no definitive diagnostic test exists.

Delayed or absent pubertal development secondary to hypogonadotropic hypogonadism is reported to affect one in 7500 males and one in 70,000 females.[551] Investigations of families with this disorder have identified mutations in several genes. Phenotypic heterogeneity of clinical presentations has become evident. Thus, there is variation regarding sense of smell, non-reproductive clinical features, patterns of endogenous GnRH secretion, and severity of clinical features. Kallmann syndrome is the eponym used to describe anosmic/hyposmic hypogonadotropic hypogonadism. Detailed investigation of human fetuses with *KAL1* mutations verified the extra-CNS embryonic origins of GnRH neurons and the failure of olfactory and GnRH neurons to migrate to the CNS.[552]

Mutations associated with the hyposmia/anosmia phenotype include genes discussed above that influence GnRH neuron migration as well as those that influence GnRH neuron function and action. Specifically, mutations have been identified in the *KAL-1, FGFR1, FGF8, KISS1, KISS1R*, and *GnRHR* genes.[23,24,171,484,553,554] Non-reproductive clinical features can accompany specific gene defects. Synkinesis is typically associated with *KAL1* mutations, but can occur with mutations in *PROK2/PROKR2* genes. Cleft lip/palate was associated with *FGF8, FGFR1*, and *CHD7* mutations.[555] Some individuals with hypogonadotropic hypogonadism carry genetic variants in two or more different genes and represent examples of oligogenic disorders.[556]

Curiously, approximately 10–15% of individuals diagnosed to have hypogonadotropic hypogonadism show reversal of the condition in early adulthood following replacement therapy with sex steroids. This reversibility has been described in association with mutations in the *CHD7, GnRHR, FGFR1, TAC3*, and *TACR3* genes.[557,558] It has been suggested that plasticity of the neuronal network governing GnRH pulsatility exists and is influenced by exposure to sex steroids. Indeed, patients diagnosed to have constitutional delay may show onset of puberty following sex steroid treatment to "kickstart" the pubertal process. Reversal in patients with loss of function mutations in the NKB signaling pathway is of particular interest (see Ref. 3) because as described earlier this pathway has been posited to comprise an integral component of the hypothalamic GnRH

pulse generator. In this regard, it is tempting to speculate that other tachykinins may be able to compensate for the abrogation of NKB signaling. Consistent with this idea is the recent finding that NKB may activate kisspeptin neurons in the arcuate nucleus of the mouse via multiple tachykinin receptors.[559]

CONCLUSION

The study of the neuroendocrine mechanisms regulating the onset of puberty in primates has been restricted almost exclusively to the infraorder Catarrhini, and primarily to Old World monkeys and man. In such highly evolved primates, puberty may be viewed as manifesting itself as a result of the cascade of physiological and behavioral processes that are set in motion by the reawakening of a restrained, but functional hypothalamic GnRH pulse generator. In males, maturation of the hypothalamic GnRH pulse generator appears to be completed during fetal development, but in the female the capacity of this system to generate an adult frequency of one pulse per hour may not be completed until after birth. The protracted hiatus in gonadotropin secretion from late infancy until puberty is viewed to result from an action of a neurobiological brake that is imposed on the GnRH pulse generator during juvenile development. The application of this brake on GnRH pulse generation, which may be achieved by a decrease in excitatory input, an increase in inhibitory input, or a combination of both, is achieved by extragonadal mechanisms. The rising circulating concentrations of sex steroids produced as a result of gonadarche, however, retard the pubertal resurgence in pulsatile GnRH release. Thus, the tempo at which puberty unfolds once it has been initiated is dictated by an interplay of central and gonadal factors, together with metabolic cues and environmental factors.

The components of the neurobiological brake on the hypothalamic GnRH pulse generator remain poorly understood. Although the kisspeptin—KISS1R signaling pathway has attracted unprecedented attention in this regard since the last edition of this book, it is likely that this neuropeptide's role in the onset of puberty is that of a GnRH pulse-generating signal, and as such is slave to the upstream components of the neurobiological brake, which dictates the "on-off-on" pattern of GnRH pulsatility during postnatal primate development. Although an increase in GABA signaling and a decrease in glutamate tone in the MBH appear to be a component of the brake, the neurobiological switches that first restrain and then reactivate GnRH pulse generator activity in the infant–juvenile and juvenile–pubertal transitions, respectively, remain poorly understood, and the developmental cues that time the activation of these switches remain a fascinating mystery. The notion that these developmental cues are governed by a program of sequential expression of "puberty" genes or gene networks in the hypothalamus (a puberty clock) in analogy to the genetic control of embryonic development has been fueled by the emergence of high throughput technologies to sequence genomes and transcriptomes. At the present time we prefer the alternative idea that the two switches controlling the postnatal activity of the GnRH pulse generator are controlled by a central growth-tracking device, termed a somatometer, that is able to respond to peripheral cues reflecting the status of somatic development. In either model, many hypothalamic genes are likely to be involved and their expression will be governed by both classical transcriptional regulation and by DNA methylation.

Also, regardless of the model, puberty will only be manifest once the neurobiological brake has been lifted at the end of juvenile development if metabolic status, as reflected by such peripheral signals such as leptin, is not compromised by undernutrition or disease. Although the actions of metabolic signals are in many cases essential for puberty onset, they should be viewed as permissive and not as components of the timing mechanism that activate the pubertal switch that leads to reaugmentation of the hypothalamic GnRH pulse generator.

There are obviously major hurdles to overcome before the mystery of puberty in primates is resolved. Perhaps most importantly, the neurobiological mechanisms underlying GnRH pulse generation need to be fully elucidated before the developmental control of this system can be established. In this regard, the hypothesis that KNDy neurons in the arcuate nucleus represent the hypothalamic GnRH pulse generator is attractive, but at the present time that is all that it is: a hypothesis. Species differences in the postnatal development of the GnRH pulse generator need to be recognized and models sought that will address the fundamental questions related to the timing of the onset of human puberty, namely what are the neurobiological mechanisms underlying the switches that first turn off pulsatile GnRH secretion during infancy and later reactivate this pattern of neuroendocrine activity to trigger puberty, and what is the biological system that has evolved in Old World monkeys, apes and humans to dramatically separate these two critical postnatal events in these species.

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CHAPTER

33

Neuroendocrine Control of Gonadotropin Secretion: Comparative Aspects

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INTRODUCTION

One objective of this new edition of *Knobil and Neill's Physiology of Reproduction* is to increase the consideration of species similarities and differences in each of the chapters. However, it was also recognized that the chapters describing the neuroendocrine mechanisms underlying puberty and ovarian cycles in rats, sheep, and humans and other primates were particularly useful in previous editions and could not realistically be combined. Therefore, these species-specific chapters have been retained (with the chapters on rats being expanded to include mice), and this new chapter has been added to provide a brief species comparison of the neuroendocrine mechanisms controlling gonadotropin secretion. This chapter will focus primarily on the female because species differences in function of the hypothalamo–pituitary–testicular axis are less striking and have not been as well studied as those in females. Because many of these concepts are firmly established, much of the material covered will be more distant than topical, but I will touch on current controversies and overlooked conundrums where appropriate. I will also not duplicate detailed discussions of mechanisms underlying puberty and ovarian cycles in rats, sheep, and primates (readers are referred to the species-specific chapters for this information), and include only selected references from these species (a more comprehensive bibliography being available in those chapters); more complete citations will be provided when discussing other species, primarily rabbits and guinea pigs.

Before considering the neuroendocrine control of gonadotropin secretion, it is useful to review the major similarities and differences in the pattern of ovarian function among species. In general, control of gonadal function is more complex than that of other endocrine tissues because the gonads produce two products: hormones and gametes. Moreover, within reproduction, the female reproductive system is more complicated than that of the male because the female has two distinct reproductive functions: (1) make gametes and ensure they are fertilized (analogous to that of the male), and (2) maintain the developing young in utero throughout pregnancy. These two functions require different endocrine environments, with estradiol (E_2) from the follicle being most important for the former, and progesterone from the corpus luteum (and placenta) for the latter. The second system becomes fully active only during pregnancy, but in many mammals progesterone secretion from the corpus luteum (CL) occurs during the normal cycle to help prepare the uterus for implantation if fertilization occurs. Ovarian cycles arose in part because these two systems must function sequentially: a mature ovum (ova) must develop before pregnancy can occur.

This sequential function is clearly evident in the phases of the menstrual cycle in women and most other primates, which can be easily divided into 2-week-long follicular and luteal phases.[1] During the follicular phase, a follicle (and ovum) develops and produces increasing amounts of E_2 (Figure 33.1(A)). The follicular phase ends with ovulation of the mature ovum (which is accompanied by a precipitous fall in E_2), and the remnants of the follicle in the ovary differentiate into the CL, initiating the luteal phase. As the CL grows, progesterone (and E_2 in women) concentrations increase to a peak about a week later. If implantation does not occur, the CL begins to die in the latter part of the luteal phase, with the subsequent fall in progesterone (and E_2) inducing menstruation, which signals the start of the next follicular phase. Although there are thus clear follicular and luteal phases, it is important to note that follicular development lasts longer than 2 weeks in primates (Chapter 28),[5] so it is not confined solely to the follicular phase preceding ovulation.

Ovarian cycles in other mammals can, in general, be divided into three additional groups based on the duration of the cycle. One group, typified by domestic animals (cow,

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sheep, and pig) and guinea pigs, has a luteal phase similar in length to that of primates (13–16 days), but a relatively short 3–6-day follicular phase; thus, ovarian cycles last between 17 and 21 days (Figure 33.1(B)), depending on the species. In these species, considerable follicular development occurs during the luteal phase, often evident as two to three follicular waves, but the final stages only occur after luteolysis. The most obvious outward sign of ovarian cycles is the occurrence of estrous behavior, which is coupled to ovulation. Thus these are referred to as estrous cycles, and the day of estrus is taken as day 0 of the cycle. The third group of animals, which consists largely of rats, mice, and hamsters, has shortened both the follicular phase and luteal phase to 1 or 2 days and has estrous cycles that are 4 or 5 days in length.[3] The 4 days of the estrous cycle are usually referred to as estrus (ovulation and estrous behavior), metestrus (increasing progesterone), diestrus (falling progesterone and increasing E_2), and proestrus (peak E_2). There is also a peak in follicular progesterone secretion on the afternoon of proestrus (not shown in Figure 33.1) that induces estrous behavior early the next day. In these species, a neural signal induced by coitus prolongs the lifespan of the CL to 9–14 days (Figure 33.1(C)). This condition, known as pseudopregnancy because it can be induced with a vasectomized male, provides sufficient progesterone to allow implantation if fertilization occurs. The final large group of mammals, exemplified by rabbits, domestic cats, mink, and ferrets, has eliminated the luteal phase completely unless coitus occurs.[6,7] Females of these species that are isolated from males show waves of follicular development and sufficient E_2 secretion to produce estrous behavior, but never ovulate. Mating of estrous females, even with sterile males, will induce ovulation and a subsequent luteal phase (Figure 33.1(D)) lasting 14–40 days, depending on the species. In some species (e.g., cats and rabbits), pseudopregnancy is terminated if implantation does not occur, but in others (e.g., ferrets) the duration of pseudopregnancy is the same as pregnancy. Because ovulation is induced by a neuroendocrine reflex triggered by coitus, these animals are referred as "reflex ovulators" to distinguish them from the other three categories, all of which are "spontaneous ovulators."

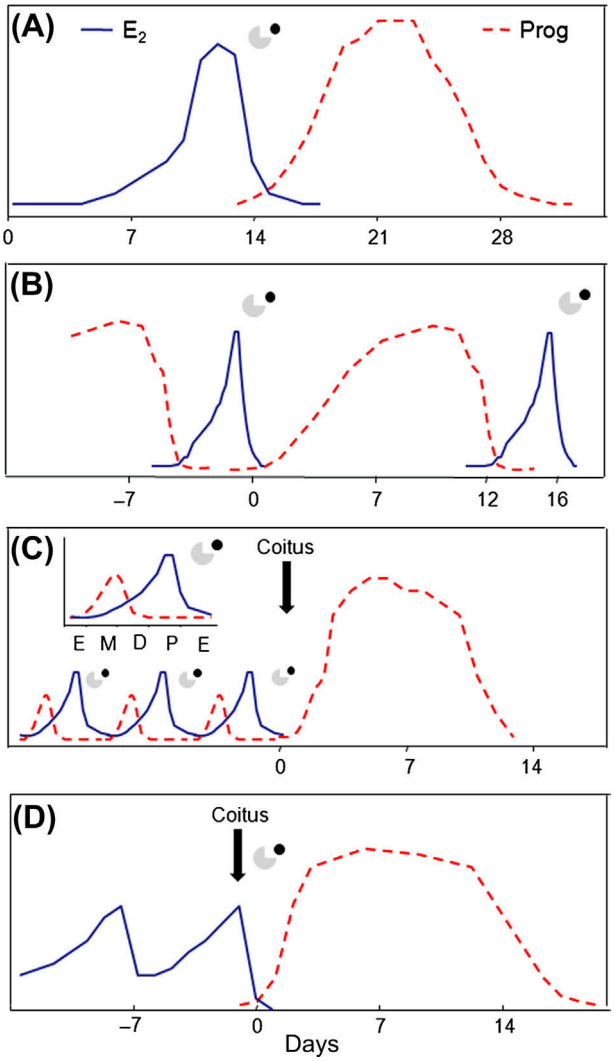

FIGURE 33.1 Patterns of follicular estradiol (E_2: solid lines) and luteal progesterone (Prog: dashed lines) to illustrate ovarian cycles of women and other primates (panel A); domestic animals and guinea pigs (panel B); mice, rats, and hamsters (panel C); and reflex ovulators (panel D). Ovulation (ovary–ovum symbol) occurs on day 14 of the primate menstrual cycle and day 0 of the estrous cycle in other mammals. In most rodents, and all reflex ovulators, coitus (arrow) is needed to induce a full-length luteal phase. Also note that in some species (e.g., rodents and primates), the follicles also secrete progesterone, while in others (e.g., some primates, and rabbits) the corpus luteum also produces E_2; these steroid patterns have been omitted from this figure in the interests of simplicity. Hormonal patterns taken for panels A–D are based on data in women,[1] sheep,[2] rats,[3] and rabbits,[4] respectively.

HISTORICAL DEVELOPMENT OF CONCEPTS FOR FEEDBACK CONTROL OF GONADOTROPIN SECRETION

Despite the diversity in ovarian cycles among mammals, the underlying events are controlled by the same basic feedback relationships between ovaries and the hypothalamo–hypophysial unit. Our understanding of these complex feedback relationships developed gradually over half a century, in parallel with the development of models to account for ovarian cycles. Indeed, much of the experimental work during this period appears to have been driven in part by a search for the mechanisms responsible for cyclic ovarian function.

Early Models

The first endocrine model for cyclic ovarian function was proposed by Loeb in 1910, based on observations

that removal of the CL of the guinea pig advanced ovulation, while prolongation of luteal function in pregnancy delayed it.[8] He thus proposed that a hormone from the CL "prolongs… the interval between two successive ovulations by preventing rupture of the follicles" and that spontaneous luteolysis in nonpregnant animals allows a new cycle to begin. While this provided a simple explanation for ovarian cycles, its basic assumption that follicular development and rupture occurred independently of extra-ovarian influences was soon disproven by the discovery of the gonadotropic action of the pituitary.

In 1927, Smith and Engle[9] demonstrated that hypophysectomy caused gonadal atrophy that could be prevented by implants of anterior pituitary tissue, and suggested that "the hypothesis of the periodic liberation of gonadal-stimulating hormone of the pituitary may explain the periodic ripening of groups of follicles more satisfactorily than any other heretofore advanced."[9] These results immediately shifted attention to the role of the pituitary and triggered studies leading to the first description of the inhibitory actions of gonadal hormones on gonadotropin secretion by Moore and Price.[10] These workers proposed that a negative feedback loop existed between the gonads and the pituitary. A negative feedback loop in which one hormone (i.e., gonadotropin) stimulates secretion of a second hormone (i.e., E_2), which in turn inhibits secretion of the first hormone, is the most common control system in endocrinology because it is designed to maintain homeostasis in the system. However, Moore and Price proposed that appropriate lag times in the negative feedback loop between the ovaries and the pituitary could account for ovarian cycles: "Starting with hypophysial secretion of gonad-stimulating substance, the ovary is stimulated and follicles grow and mature. During follicular activity large amounts of oestrin are liberated… [which] leads to depression of the hypophysis, causing it to lag in the secretion of the gonad-stimulating substance, which accounts for cessation of further follicular development. When oestrin is no longer produced in large amounts… the depressing action upon the hypophysis ceases and it again responds by a secretion of more gonadal-stimulating hormone and the cycle is repeated."[10] We now know that this feedback relationship would reach a steady state rather than cycle; in fact, this was first demonstrated in 1936 by Pfeiffer, who induced just such an equilibrium by "masculinizing" female rats with testicular implants during the neonatal period.[11] When these females matured, they failed to cycle, instead showing a condition known as "constant estrus," with large follicles in the ovary, and vaginal histology indicative of continuous E_2 secretion. Nevertheless, the basic concept of a negative feedback loop proposed by Moore and Price is still applicable to the relationship between testes and hypophysis, although of course the importance of the hypothalamus and both testosterone and inhibin is now incorporated into it. In fact, this negative feedback theory for ovarian cycles was short-lived largely because it was overtaken by the simultaneous discovery that the pituitary produced two hormones: one that stimulated follicular activity and E_2 secretion (follicle-stimulating hormone (FSH)), and another that induced ovulation and luteinization (luteinizing hormone (LH)) and thus favored progesterone production.[12] This discovery, in turn, led to the first long-lived model for the ovarian cycle.[13]

The Sequential Secretion of FSH and LH Model

One logical inference from the proposed actions of FSH (follicular development and E_2 secretion) and LH (CL formation and progesterone secretion) is that FSH is important for the follicular phase and LH for the luteal phase of the cycle. Moreover, two new lines of evidence provided a possible explanation for the sequential secretion of FSH and LH during the cycle. First, a negative feedback relationship between E_2 and FSH was supported by reports that: (1) ovary-intact rats, parabiotically connected so that blood from an ovariectomized (OVX) animal was transferred into them, showed constant estrus suggestive of elevated FSH release in the OVX rat[14]; (2) low doses of E_2 inhibited follicular development in immature rats[15]; and (3) urine from postmenopausal women with low E_2 contained FSH-like bioactivity.[16] Second, several studies provided evidence that E_2 stimulated LH secretion. Namely, injection of high doses of estrogen to immature rats[17] and seasonally anovulatory sheep[18] induced formation of CL; since LH was responsible for luteinization, these workers suggested a stimulatory action of E_2 on LH release. In contrast, progesterone administration blocked ovulation in rats,[19] guinea pigs,[20] and rabbits.[21] These data supported the hypothesis that progesterone inhibited LH release, and they would eventually lead to development of the contraceptive pill 20 years later.[22]

In 1939, Fevold[13] synthesized these new observations into the following model for the ovarian cycle: at the beginning of the follicular phase, FSH concentrations are high and initiate a wave of follicular development. As the follicles grow, the increasing serum E_2 inhibits FSH secretion and stimulates LH secretion. Ovulation and luteinization occur once LH release is dominant. During the luteal phase, the increasing progesterone and low E_2 result in gradual decreases in LH (high progesterone) and increases in FSH (low E_2), so that at luteolysis a new wave of follicular growth can begin. Although Fevold recognized that this model might not apply to rabbits and guinea pigs,[13] it appears to have been generally accepted, and it became the dominant model for the next two decades.

Over that period, several lines of evidence undermined the basic assumptions of this model. First, as more purified LH and FSH preparations were developed, it

became evident that LH as well as FSH were needed for E_2 secretion from the follicle.[23] Second, Everett demonstrated that the properly timed administration of progesterone to rats could induce ovulation,[24] with acute progesterone treatments being stimulatory and chronic treatments inhibitory. Third, careful studies demonstrated that E_2 had two effects on LH secretion, with low doses being inhibitory and high doses stimulatory.[25] At the same time, interest was growing in the possible role of the brain in control of gonadotropin secretion. Early work had clearly demonstrated a role for the nervous system in reflex ovulation in rabbits[26] and provided indirect evidence for a hypothalamic "sexual center" in rats (see the discussion in Sawyer et al., 1949[27]). However, it wasn't until the late 1940s, when Harris and others established the functional importance of the hypophysial portal circulation,[28] that studies on the neural control of ovarian function began in earnest. One of the earliest of these was a pivotal set of studies that ultimately led to the current model for ovarian cycles.

The Tonic and Surge Secretion Model

This model postulates that a relatively constant, or "tonic," secretion of FSH and LH is responsible for follicular growth and E_2 secretion and, in turn, is regulated by the negative feedback action of this steroid. Ovulation and luteinization are the results of a surge of gonadotropin (LH) superimposed on this basal secretory rate; in reflex ovulators the LH surge is triggered by coitus, while in spontaneous ovulators it is initiated by high E_2 concentrations at the end of the follicular phase. Tonic secretion occurs in males and females, whereas the LH surge only occurs in females. The ability of E_2 to induce the LH surge in spontaneous ovulators became known as the positive feedback action of this steroid to distinguish it from the negative feedback action of E_2 on tonic LH and FSH secretion, although it is technically not a positive feedback loop. A positive feedback loop consists of two elements (i.e., LH and E_2), both of which stimulate the other, and is inherently unstable because once initiated it leads to continuous increases in both elements until an outside force breaks the feedback loop. Although tonic LH secretion stimulates E_2 secretion, the LH surge inhibits E_2 secretion, while increasing progesterone secretion, from the preovulatory follicle.

In 1949, Everett, Sawyer, and Markee laid the foundation for this model in a paper examining the possible role of adrenergic and cholinergic transmission in ovulation.[29] In this work, they demonstrated that receptor antagonists (dibenamine and atropine, respectively) blocked ovulation in rats if given before 1400 h on proestrus, but were ineffective if given at 1600 h (Figure 33.2). They thus concluded that a neurogenic signal from a hypothalamic "sexual center" on the afternoon

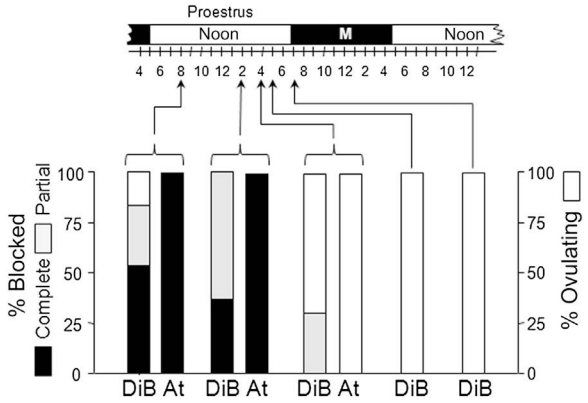

FIGURE 33.2 Administration of the α-adrenergic receptor antagonist dibenamine (DiB) and the cholinergic receptor antagonist atropine (At), at or before 1400 h of proestrus, blocks ovulation in rats. If given at 1600 h or thereafter, these drugs have little or no effect on ovulation. M: midnight. *Source: Data redrawn from Everett et al.*[29]

of proestrus triggered the preovulatory discharge of LH in rats. They also suggested, based on earlier work, that elevated estrogen initiates the "ovulation stimulus" from this center, although progesterone may synergize with E_2 under some circumstances. In a subsequent study, using pentobarbital blockade, they established that there was a "24 h periodicity in the 'LH-release apparatus' of the female rat,"[30] setting the foundation for current work on the circadian control of this system in rodents.[31]

As they had already demonstrated that dibenamine and atropine blocked ovulation if given shortly after coitus in rabbits,[32] this work in rats established a common link between control of ovulation in reflex-ovulating and spontaneously ovulating species. In addition, by limiting the time of LH release to a short period in the afternoon of proestrus, the data strongly indicated that the signal for ovulation in the rat, as in the rabbit, was an abrupt discharge of LH from the pituitary. Importantly, these authors extended the analogy between these two species by comparing rabbits to rats in constant estrus: "one direct cause of such aberrant occurrences is the failure of the hypothalamus to discharge an adequate stimulus to the hypophysis. As a result, the hypophysis continues more or less steadily to secrete only small amounts of gonadotrophin, which are nevertheless sufficient to maintain estrogen secretion. This steady state is normal in such species as the rabbit," and "the steady secreting male type is determined [in rats] by early action of androgen during infancy."

Although this model contained all the basic elements of the current tonic and surge model for the control of gonadotropin secretion, it was slow to gain acceptance. One major milestone in its progress was the work by Barraclough and Gorski[33] in 1961 on the site where neonatal androgen acts to disrupt ovulation. Although their experimental conclusion that androgen blocked the ability of electrochemical stimulation of the preoptic area (POA)

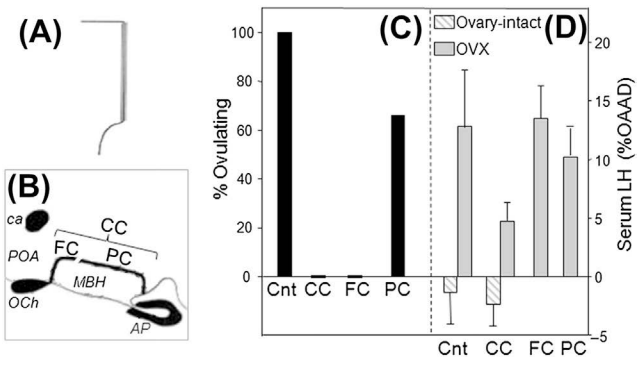

FIGURE 33.3 Effects of different neural de-afferentation procedures on ovulation in ovary-intact rats and serum LH in OVX rats. Panel A: schematic of Halasz knife that was lowered down between the two hemispheres to perform de-afferentations. By combining rotation of the knife 90° so the blade is parallel to the third ventricle and moving it in the anterior–posterior plane, the cuts in panel B were performed. Panel B: Illustration of three types of knife cuts on a parasagittal schematic of the rat hypothalamus. Complete cuts (CC) severed all connections to the mediobasal hypothalamus (MBH), while frontal cuts (FC) severed inputs from the preoptic area (POA) and posterior cuts (PC) severed inputs entering laterally and posteriorly. Effects of cuts on ovulation rate (panel C) and on tonic LH secretion (panel D), illustrated as mean (±SEM) serum LH concentrations in samples collected from OVX (shaded bars) and ovary-intact (striped bars) rats during the morning; Cnt: controls. Note that LH was measured using the ovarian ascorbic acid depletion bioassay. Serum from ovary-intact rats produced a slight (nonsignificant) increase in ascorbic acid content of the ovaries, which is plotted as a negative value. Panels A, B, and C taken from a review by Haslaz[35]; panel D is based on data presented in Halasz and Gorski.[36] AP: anterior pituitary; ca: anterior commissure; OCh: optic chiasm.

to induce LH secretion was later proved to be incorrect,[34] the hypothesis of "dual hypothalamic control of adenohypophysial gonadotropin secretion," with the arcuate–ventromedial nuclei of the mediobasal hypothalamus (MBH) controlling the "tonic discharge of gonadotropins in sufficient quantity to maintain estrogen production" and the POA producing the "cyclic discharge of gonadotropin to cause ovulation," was conceptually important. Moreover, it soon received strong support from the neural de-afferentation studies of Halasz, which clearly demonstrated that the MBH was important for tonic secretion, while input from the POA was essential for ovulation (Figure 33.3).[35–37] By placing control of tonic and surge secretion in two different hypothalamic areas, these studies provided a concrete visualization of the model that facilitated its general acceptance. The application of bioassays to the measurement of gonadotropins (primarily LH using the ovarian ascorbic acid depletion assay) in the 1960s provided further support for this model, which was formalized in its present form by Schwartz toward the end of this decade.[38] It is interesting to note that this model was essentially fully developed before radioimmunoassays (RIAs) were used to measure circulating gonadotropins and ovarian steroids, which is a tribute to the careful experimental designs and interpretations of these early workers. The subsequent RIA measurements of LH and FSH concentrations during the ovarian cycles of several species,[39,40] including humans,[39] and the application of the concept that different mechanisms control tonic and surge secretion of gonadotropins to the rhesus monkey[41] demonstrated its general applicability and solidified its acceptance.

While the tonic and surge model for control of gonadotropin secretion is now generally applied to all mammals, it should be kept in mind that it has been rigorously tested in only a few of these. In this regard, the estrous cycle of the mare is a cautionary example. At a superficial level, it closely resembles that of other domestic animals, with a length of 22 days divided into a 15–17-day luteal phase and 5–7-day follicular phase.[42] However, closer examination of the preovulatory LH surge indicates a marked difference from other animals in this group. It is prolonged, lasting about 5 days, and of relatively low amplitude, with peak levels only fivefold (compared to 50- to 100-fold increases in rats, sheep, and primates) higher than basal concentrations.[42,43] Moreover, follicular ablation studies demonstrate that it is not induced by increasing E_2 during the follicular phase, but is more likely caused by a withdrawal of steroid negative feedback due to luteolysis.[43] Thus, this ovarian cycle may depend more on changes in negative feedback, as first suggested by Moore and Price,[10] than on the differential control of tonic and surge secretion.

MODIFICATIONS IN THE MODEL: SPECIES DIFFERENCES IN GONADAL FEEDBACK

One apparent deficiency in this model is the lack of any role for progesterone beyond its ability to inhibit the preovulatory LH surge, which is evident in all species. Indeed, the model focuses much more on events during the follicular phase than on those in the luteal phase, most likely because it was based largely on work in rats, a species that does not have a functional CL in the absence of coitus. It was also recognized that there are major species differences in control of luteal function, with prolactin being the primary luteotropin in rodents, but LH performing this role in most other species (Chapter 23).[44] Moreover, in many species in which LH is luteotropic, uterine prostaglandin-$F_{2\alpha}$ is the key determinant of luteal lifespan, so the feedback actions of progesterone were thought to be of little importance. However, with the availability of RIAs and the development of physiological replacement regimens, two additional feedback actions of progesterone were identified, although the importance of these varies among species.

Control of the LH Surge: Stimulatory Actions of Progesterone

As noted in this chapter, earlier evidence in the rat indicated that progesterone could act acutely to induce ovulation[24]; later measurements of endogenous progesterone secretion indicated that it increased at the time of the LH surge in many species and raised the possibility that this action might be physiologically relevant. In rats,[45] guinea pigs,[46,47] monkeys,[48] and women,[49] the preovulatory LH surge induces an increase in progesterone secretion from the ovulating follicles, although the increment is modest in primates compared to the dramatic increase in rats and guinea pigs. This response to the LH surge is not seen in sheep[50] (although it does increase progesterone levels within the follicle), other domestic animals,[40] and reflex ovulators,[6] except for the rabbit, in which the primary steroid released is 20α-hydroxyprogesterone.[51]

While acute stimulatory effects of progesterone on LH can be demonstrated in rats,[52] guinea pigs,[53,54] rabbits,[55,56] monkeys,[57] and humans,[58,59] there are species differences in the physiological role of these endogenous progestin increments. They are not important in rabbits because ovariectomy 15 min post coitus does not alter the subsequent increment in LH.[60] Likewise, in guinea pigs,[61] sheep,[62] and rhesus monkeys,[63] exogenous estrogen alone produces normal gonadotropin surges in OVX animals. Similar replacement studies in women indicated that progesterone may produce a modest stimulation of LH secretion, and a more dramatic increase in FSH secretion, during the surge.[58,59] In rodents, the stimulatory actions of progesterone appear to be important in amplifying the LH surge.[64,65] It should also be noted that the chronic inhibitory actions of this progesterone increment limit the LH surge to proestrus,[66] instead of occurring daily as is seen in estrogen-treated OVX rats.[64] Interestingly, the stimulatory action of progesterone in rodents may well be one biological consequence of a key role for progesterone receptors (PRs) because they appear to be necessary for the preovulatory gonadotropin surge in rodents, even when it is induced by E_2.[67,68] Whether PR signaling is critical in other species has yet to be determined, but this does not seem to be the case for the ewe.[69] Thus in most, but not all, species, progesterone can synergize with E_2 to facilitate the preovulatory gonadotropin surge, but the physiological importance of this action varies across species.

Control of Tonic LH Secretion: Inhibitory Actions of Progesterone

Careful studies with physiological hormone replacement clearly demonstrate that progesterone can synergize with low E_2 levels to inhibit tonic LH secretion in rats,[70] sheep,[71] monkeys,[72] and humans.[73] Not surprisingly, this appears not to be the case for reflex ovulators[6]: E_2 is sufficient to hold LH secretion in check in OVX ferrets,[74] cats,[75] and rabbits,[76] and tonic LH concentrations during pseudopregnancy are similar to those in estrous rabbits.[4] In those spontaneous ovulators in which synergistic effects occur, it is also clear that progesterone usually has little or no effect in the absence of E_2, and the responses to the negative feedback actions of steroids change with time after OVX. These data, together with observations that LH concentrations during the cycle are not obviously negatively correlated with progesterone in monkeys[41] and rats,[77–79] supported the conclusion that E_2 is the primary negative feedback steroid in these species. In contrast, Karsch et al.[71,80,81] presented strong evidence that progesterone is the primary negative feedback hormone during the estrous cycle of sheep. They[82] and others[83] also proposed that the fall in circulating progesterone concentrations at luteolysis, by allowing a sustained increase in tonic LH secretion that drives the preovulatory E_2 rise needed to induce the LH surge, was the key event controlling the timing of ovulation in this species. Interestingly, some of the strongest support for this hypothesis was analogous to the early work of Loeb in the guinea pig[8]: removal of the CL caused premature initiation of these events, including ovulation, while prolongation of elevated progesterone delayed them.[80,83] Thus, there are species differences in which steroid is the primary negative feedback hormone during the ovarian cycle: it appears to be E_2 in reflex ovulators, rats, and primates, but progesterone in sheep, cows, and probably guinea pigs.

Control of Tonic LH Secretion: Steroid Inhibition of Episodic LH Secretion

The advent of RIAs soon led to the discovery that tonic LH secretion was not the "steady" continuous type of release originally envisioned, but instead was secreted episodically. The initial report of episodic LH secretion in OVX monkeys[84] was soon extended to include a large number of species and endocrine conditions.[85] When the negative feedback control of episodic LH secretion was examined, an interesting pattern emerged: in primates and domestic animals, progesterone inhibited LH pulse frequency, while E_2 inhibited pulse amplitude.[85–87] These actions were evident when pulse patterns in ovary-intact females were compared with those in OVX animals or postmenopausal women (Figure 33.4) and provided a simple explanation for differential patterns of episodic LH secretion during the ovarian cycle of these species, with low-amplitude high-frequency pulses in the follicular phase, and high-amplitude low-frequency pulses in the luteal phase.[88–90] The two exceptions to this general pattern are reflex ovulators and rats. LH pulse frequencies are similar in estrous and pseudopregnant rabbits,[4] and E_2 inhibits LH pulse frequency in OVX rabbits[76] and ferrets.[74]

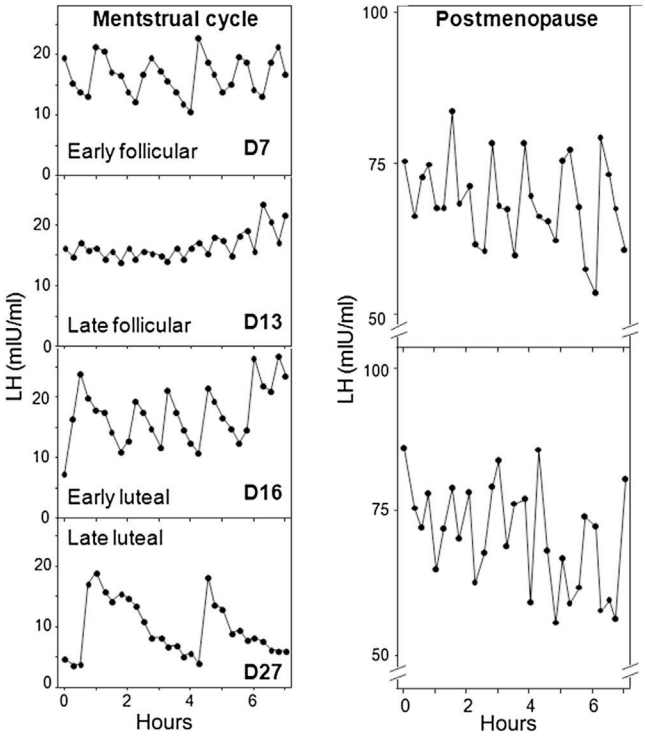

FIGURE 33.4 LH pulse patterns on four different days of the menstrual cycle (left) and from two postmenopausal women (right). Note that y-axes on the right have been adjusted to start at 50 mIU/ml so that pulse amplitudes can be directly compared with those on the left. *Source: Redrawn from Yen et al.[88] with permission from the Endocrine Society.*

In rats, there are no changes in episodic LH secretion during most of the 4-day estrous cycle except on the morning of estrus when few,[77] or no,[78,79] LH pulses are observed. When progesterone negative feedback on LH pulses is examined in rats, the primary effect appears to be on LH pulse amplitude,[91–93] although there is strong evidence that the proestrus surge in progesterone secretion suppresses pulsatile LH secretion on the morning of estrus.[94] Inhibitory effects of E_2 on either LH pulse frequency[91,95] or amplitude[78,91,95] have been reported, probably due to differences in steroid concentrations or treatment regimens. For example, careful comparison of the effects of physiological E_2 concentrations indicated that the higher levels on proestrus inhibited LH pulse frequency,[95] while lower concentrations seen on diestrus-2 did not.[93] The species difference in progesterone negative feedback may reflect the absence of a prolonged luteal phase in the rat, as progesterone (when combined with E_2) strongly inhibits LH pulse frequency on days 6 to 8 of pregnancy.[96]

One fairly consistent but still unexplained observation from these studies is that steroid replacement in OVX animals appears to be more effective than the same concentration of steroids produced by the ovaries, at least in rats and monkeys. Thus, the combination of physiological E_2 and progesterone replacement completely suppresses LH secretion in OVX monkeys and rats, a phenomenon not evident in ovary-intact animals. Similarly, E_2 treatment completely eliminates episodic LH secretion in OVX animals,[78,97,98] even though clear LH pulses are evident in the presence of the same E_2 concentrations in the follicular phase. This led to the proposal that an ovarian factor acts to ameliorate the negative feedback actions of E_2 and/or progesterone.[98] There is still no evidence as to what this factor is, and other possibilities have been suggested. For example, replacement regimens do not match the dynamic pattern of steroid secretion produced by the ovary. If such a factor exists, it is unlikely to be important in sheep since physiological E_2 and progesterone replacement results in LH concentrations indistinguishable from those in the normal ovine estrous cycle.

Control of Tonic FSH Secretion: Inhibins, Activins, and Follistatin

In many early RIA studies, LH and FSH concentrations generally changed in parallel so it was assumed that they were controlled by the same ovarian feedback mechanisms. This assumption appears to still hold for surge secretion of FSH, which is induced by the positive feedback action of E_2, supplemented by progesterone in some species. In contrast, it is now clear that tonic secretion of FSH is controlled, in part, by negative feedback actions of inhibin in many species. "Inhibin" was initially described in 1932 as a water-soluble testicular extract that inhibited castration-induced changes in the cell structure of gonadotropes.[99] Inhibin was subsequently associated with selective inhibition of FSH secretion based on positive correlations of urinary FSH, but not LH, with the degree of damage to spermatogenesis in infertile men.[100] However, this concept gradually fell into disrepute because the initial observations could not be replicated and attempts to purify inhibin were unsuccessful. Interest in inhibin was rekindled by RIAs, which demonstrated clear instances of differential secretion of FSH and LH, which included selective declines in FSH secretion (in the latter half of the follicular phase in primates and domestic animals) and selective increases in FSH release (during the peri-menopausal period in women, and "second FSH surges" in rodents and domestic animals). This ultimately led to the identification of two inhibins, inhibin A (α-β_A) and inhibin B (α-β_B), that inhibit FSH; activins (dimers of β_A and β_B) that stimulate FSH secretion; and follistatin, a binding protein that inactivates activin.[101,102] It was also soon recognized that these proteins are produced in a variety of tissues, including the anterior pituitary, and play important physiological roles beyond regulation of FSH secretion.

Although the ovaries produce inhibin A and B, activins, and follistatin, inhibins are probably the only important hormonal product because most of the circulating activin and follistatin form an inactive complex and the free forms show little, if any, variation that correlates with changes in FSH secretion.[103] Thus, paracrine actions of activin and follistatin at the pituitary are probably much more important to control of FSH secretion than their endocrine actions from the ovary. While inhibin is now recognized as an important inhibitory hormone, there are species differences in the relative roles of inhibin and ovarian steroids in the control of tonic FSH secretion. In rats[104] and sheep,[105] inhibin plays an important negative feedback role because physiological concentrations of progesterone and E_2 produce normal levels of LH, but only partially suppress FSH concentrations in OVX animals. Moreover, passive immunoneutralization of inhibin in rats[106] and cows[107,108] produces dramatic increases in FSH secretion that are often greater than those seen when antibodies to E_2 are administered. Thus, a negative feedback loop between follicular inhibin and FSH secretion controls follicular development and ovulation rates in these species.[109] The importance of inhibin in primates is less clear; physiological replacement with ovarian steroids in OVX monkeys maintains normal concentrations of both FSH and LH,[72] but similar treatments in women with premature ovarian failure produce normal LH, but elevated FSH, concentrations.[110] It is clear that a negative feedback loop between the developing follicles and FSH secretion is important to the control of follicular development in primates,[109] and some evidence points to an important role for inhibin,[111] but the preponderance of data (reviewed by Zeleznik and Plant[1]) indicates that E_2 is the primary negative feedback hormone in the follicular phase of the menstrual cycle.

There are species differences in the ovarian sources of inhibin A and inhibin B and thus differences in secretion patterns of these proteins. In rats and primates, follicles secrete similar amounts of both inhibins, while in sheep,[112] pigs,[113] cows, and horses[109] only inhibin A is secreted in biologically important amounts. In the species whose ovaries produce both inhibins, inhibin B originates primarily from small antral follicles, while inhibin A comes from preovulatory follicles.[114–116] This results in differential secretion of the two inhibins as the follicular phase progresses,[109,117,118] which may be useful in monitoring follicular development but is unlikely to be important to control of FSH because inhibin A and inhibin B have similar biological effects.[119] A more important species difference may be that the corpora lutea of Old World monkeys[115] and women[116] produce inhibin A, while those in most other species do not.[102,112,113] It is tempting to speculate that luteal secretion of inhibin A holds FSH release at minimal levels in these primates, thus preventing the follicular waves seen in other species during the luteal phase, and producing the relatively long follicular phase seen in primates. However, inhibin A does not appear to play such a role in monkeys because antibodies against this peptide did not alter FSH secretion in the luteal phase[120] and exogenous inhibin A failed to prevent the FSH increase that follows luteolysis in stump-tailed macaques.[121] Instead the combination of E_2 and progesterone likely accounts for changes in FSH concentrations during the luteal phase in primates.[1] In light of this conclusion, the production of E_2 by the primate CL, but not by the CL of domestic animals, may account for species differences in follicular development during the luteal phase, and thus the longer follicular phase of the menstrual cycle.[122]

NEUROENDOCRINE SYSTEMS MEDIATING STEROID FEEDBACK

For many years now, one major focus of reproductive neuroendocrinologists has been to identify the components within the hypothalamo–pituitary unit responsible for tonic and surge secretion of gonadotropin-releasing hormone (GnRH) and their control by ovarian steroids. These studies required invasive, and often complicated, experimental manipulations and have largely been limited to three species: rats, sheep, and monkeys. Therefore, in this section, I will compare the considerable information currently available on these three species with the more limited data available on humans and other species. I will first describe the effects of ovarian steroids at the level of the pituitary and then consider hypothalamic systems controlling tonic and surge secretion of GnRH.

Effects of Ovarian Steroids on the Pituitary

One enduring controversy, which has yet to be completely resolved, is the relative importance of hypophysial and hypothalamic sites of action of E_2 and, to a lesser extent, progesterone. A clear determination of the actions of ovarian steroids on the pituitary in situ is complicated because it is difficult to distinguish between direct effects on gonadotropes and indirect effects via changes in hypothalamic release of GnRH secretion. An increase in endogenous GnRH can affect the response to exogenous GnRH via several mechanisms, including an induction of GnRH receptors on gonadotropes[123] and a self-priming action, whereby a second injection of GnRH produces a larger increment than the first injection.[124] It is also clear that LH pulse amplitude is inversely related to GnRH pulse frequency[125] and directly related to GnRH pulse amplitude, and that changes in frequency can alter the relative secretion of FSH and LH, with high frequencies favoring the latter.[126,127] To eliminate possible confounding changes in endogenous GnRH secretion, workers developed

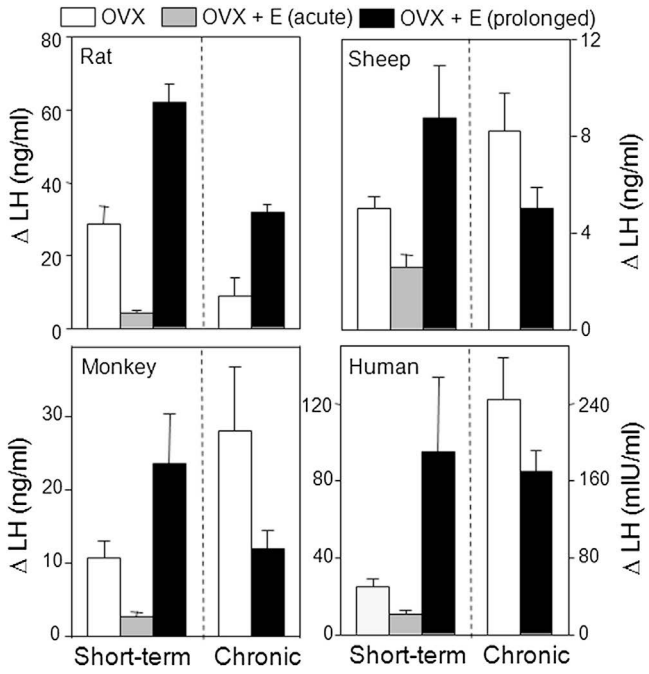

FIGURE 33.5 Effects of short-term and chronic treatment with E_2 on the pituitary response to GnRH (plotted as mean ± SEM ΔLH) in rats,[131] sheep,[129,132] monkeys,[128,133] and humans.[134,135] With short-term treatments of E_2, an initial (acute) suppression in response to GnRH is followed by stimulation of the response (prolonged treatment); in these panels, the nadir and peak responses are presented (see the text for approximate times of each). Chronic treatments with E_2 were for 48 h in rats,[131] 7 days in sheep[132] and women,[135] and 13 days in rhesus monkeys.[133] Data from sheep and short-term treatments in monkeys were collected using a hypophysial clamp approach; all other data were from animals or patients with an intact hypophysial–pituitary unit. Note that in most cases, data for short-term and chronic effects of E_2 are from different studies and cannot be directly compared.

techniques in monkeys,[128] sheep,[129] and pigs[130] to eliminate endogenous secretion by either lesioning the MBH or surgical disconnection of the MBH from the anterior pituitary so they had complete control of the exposure of the in situ pituitary to GnRH given exogenously (often referred to as a "hypophysial clamp").

Although not all studies have controlled for endogenous GnRH release, very similar patterns emerge (with one exception) when the effects of E_2 on the GnRH-induced increment in LH concentrations are examined across several species (Figure 33.5). In all cases, a short-term exposure to this steroid initially inhibits the response to GnRH, but with continued exposure to E_2 an augmented increment in LH is observed. The exact time course varies among species, being shortest in rats (nadir at 4–5 h and peak at 12 h),[131] intermediate in sheep (nadir: 6–10 h; peak: 20–28 h),[129] and longest in primates (nadir: 16–24 h[128,134]; peak: 3–4 days[128,135]). A similar pattern has been reported in cows[136] and guinea pigs,[137] but the stimulatory effects observed in the latter are fairly modest, particularly when compared to the dramatic effects of E_2 on the self-priming action of GnRH.[137] The one exception to this general pattern is the pig, in which E_2 appears to have inhibitory, but no stimulatory, effects on the pituitary.[130] It should be noted that very rapid inhibitory effects can be observed in several of these mammals with intravenous infusion of E_2,[138–141] although these studies sometimes used supraphysiological E_2 treatments. Interestingly, the latency to the stimulatory effects of E_2 on the pituitary generally corresponds to the time needed to induce an LH surge, and is positively correlated with the length of the follicular phase in these species. With more chronic exposure (i.e., at least several days) to E_2, inhibitory effects on the response to GnRH are observed in rabbits,[76] sheep,[132] monkeys,[133] and women.[135] On the other hand, the stimulatory effect observed in the rat with short-term exposure is maintained with chronic exposure to E_2 (>24 h).[131] These species differences are consistent with observations that a chronic increment in E_2 induces only a single LH surge in sheep and primates, but daily LH surges in rodents.[64]

The physiological importance of progesterone actions on gonadotropes remains unclear. These have not been as extensively studied as those of E_2 and vary considerably among species. In rats, these actions generally correlate with the effects of progesterone on LH secretion, with acute exposure (for 3–8 h) enhancing the response to GnRH, and prolonged exposure (at least 48 h) inhibiting this response.[3] In women with deficient GnRH secretion, progesterone increased LH pulse amplitude in response to exogenous GnRH pulses,[142] an action that may contribute to the elevated response to GnRH observed during the normal luteal phase.[143] In monkeys, stimulatory effects of progesterone on the pituitary have been reported,[1] but these are not reflected in an increased response to GnRH in luteal phase animals.[133] The situation is equally confusing in the sheep since both stimulatory[129] and inhibitory[144] effects of progesterone on the GnRH-induced increment in LH have been reported using similar models. Thus, in contrast to the effects of E_2 on the pituitary, which generally correlate with its negative and positive feedback actions in most species, the effects of progesterone are more variable and their physiological relevance remains unclear.

Hypothalamic Systems: General Considerations

There is evidence in most species that E_2 and progesterone also have important feedback actions in the brain to control episodic GnRH secretion. The inhibition of LH pulse frequency by progesterone clearly implies that this steroid inhibits GnRH pulse frequency, and this has been confirmed by direct measurements of GnRH. However, inhibition of LH pulse amplitude by E_2 could reflect actions solely at the pituitary, and there is considerably more controversy as to whether this steroid also inhibits GnRH pulse amplitude in some species, but clear evidence for

this in rabbits and sheep (discussed later in this chapter). It is also clear that E_2 induces a preovulatory GnRH surge in spontaneous ovulators, but there may be important species differences in the physiological significance of this positive feedback action of E_2. Furthermore, there are major species differences in the anatomical substrates and sites of action of ovarian steroids, so this section will consider the comparative aspects of each of these in some detail.

Anatomical Aspects

When considering neural aspects of these control mechanisms, it is also useful to distinguish between anatomical areas and specific neuronal phenotypes. At the outset, it should be emphasized that experiments focused on the neuroanatomy of these control systems were designed to determine the minimal neural areas required for normal function, and did not exclude other regions providing physiologically relevant modulatory input. It is thus important to remember that redundancy is one hallmark of the reproductive neuroendocrine system. This is evident in estimates of the number of GnRH neurons needed for normal episodic GnRH secretion, which range from 70–80 neurons in mice[145] to approximately 100 in sheep[146]; indeed, 1–28 GnRH neurons in hypothalamic grafts are apparently sufficient to produce episodic LH secretion in castrated male *hpg* (hypogonadal) mice.[147] Similar, if not greater, redundancy is seen in regard to ovulation. Thus, much more LH is released during the preovulatory surge than is required for ovulation,[148] and only a small fraction of the preovulatory GnRH surge is required to produce a normal LH surge, at least in sheep.[149] Similar redundancy is evident in other neural systems controlling GnRH release.[150,151] Thus, as exemplified by evidence from monkeys for a preovulatory GnRH surge that is not required for normal menstrual cycles,[152] determination of the minimal systems required for normal function is likely to exclude mechanisms that function under physiological conditions.

Neural Systems Mediating Feedback Actions of Steroids

Although a multitude of neurotransmitters have been implicated in the control of both tonic and surge secretion of GnRH (see Chapter 11),[153] a consideration of most of them is beyond the scope of this chapter. However, sufficient information is available on three of these to allow useful species comparisons: norepinephrine (NE), the endogenous opioid peptides (EOPs), and kisspeptin. This focus does not imply that these neurotransmitters are more important than others. For example, it is clear that both gamma-aminobutyric acid (GABA) and glutamate play important roles in controlling GnRH secretion, and a considerable amount of information is available on the effects of these transmitters on GnRH neural activity in mice.[153,154] However, no clear picture has emerged on their role in other species. Similarly, neuropeptide Y (NPY) appears to be important for the full preovulatory LH surge in rats[124] and rabbits,[6,7] but its physiological actions and roles in other species remain unclear. With NE, EOPs, and kisspeptin, a fairly consistent role has been identified across several species in the control of tonic and/or surge secretion of GnRH. The consideration of kisspeptin will be limited to a brief overview of species similarities and differences, with only a few references to the primary literature. A number of recent reviews on these actions of kisspeptin are available,[31,155,156] and they are also covered in detail by several chapters of this book.

Hypothalamic Control of Tonic Gonadotropin Secretion

Anatomical Sites of Action

There is a general consensus that the MBH–pituitary unit is sufficient for the control of tonic gonadotropin secretion in all species studied to date. Thus, knife cuts that partially, or completely, disrupt input to the MBH have little or no effect on tonic gonadotropin secretion in intact or OVX rats,[157–159] sheep,[160,161] monkeys,[162] or guinea pigs.[163] Some rats with complete cuts around the MBH show constant diestrous vaginal smears indicating low circulating E_2 and possibly inadequate tonic gonadotropin secretion. In one study,[157] these rats did not respond to OVX with an increase in LH secretion, but in another study[158] they did; in any case, no obvious histological differences in the location of the knife cuts were evident between rats showing constant diestrus and those with similar knife cuts showing constant estrus smears, indicating elevated E_2 secretion.[157] Because episodic LH secretion was also evident after OVX in all these studies, the neural substrates responsible for GnRH pulse generation must reside within the MBH, a conclusion consistent with the location of most electrodes that record bursts of multiunit electrical activity (MUA) that correlate with endogenous LH pulses in several species.[164–168] The conclusion that the MBH–pituitary unit is sufficient for normal episodic LH secretion is consistent with the location of GnRH cell bodies within the MBH in primates, sheep, and guinea pigs, but is at odds with the general consensus that no GnRH cell bodies are found in the MBH of rats.[153] However, a few scattered GnRH cell bodies are observed in the MBH of rats with some antibodies.[169,170] It has also been suggested that a projection from rostral GnRH cell bodies could have been missed by these knife cuts,[171] and may be sufficient to account for the GnRH immunoreactivity observed in the median eminence[159,171–173] and maintenance of episodic LH release after de-afferentation. Evidence that only a few GnRH neurons are sufficient for episodic GnRH secretion supports this possibility. However, the effects of

knife cuts that clearly severed this pathway, along with the other efferents from POA GnRH cells, on LH pulses have not been determined. Thus, this apparent paradox in rodents remains to be completely resolved.

There is less information available on the areas within the MBH that are necessary for tonic GnRH secretion. In guinea pigs, the arcuate nucleus (ARC) appears to be the critical area,[174] while in the rat, lesion data point to the anterior portion of this nucleus as critical for episodic GnRH secretion.[158,159] Interestingly, in at least one rodent study, knife cuts through the anterior arcuate largely disrupted LH pulses, but LH concentrations were still detectable.[159] Since LH pulses were augmented by transplantation of fetal MBH tissue that did not contain GnRH cells,[172] it has been proposed that non-GnRH neurons in this region help drive episodic GnRH release by an action at GnRH terminals in the median eminence.[175] The anterior region of the ARC may also be important in sheep as well, based on the effects of frontal knife cuts in this area to disrupt episodic LH secretion in OVX ewes, although damage to posterior regions of the ARC may have occurred.[160,161] It is interesting to note that these lesion data in rats and sheep are not entirely consistent with the recently proposed role for ARC kisspeptin cells in driving GnRH pulses,[176–179] since these are concentrated in the posterior, not the anterior, ARC. In contrast, lesion data in rhesus monkeys are more consistent with this hypothesis because more posterior portions of the ARC must be lesioned to completely disrupt LH and FSH secretion in OVX females.[180] Thus, another unresolved question is the nature of the neural elements in the rostral ARC that appear to be important for episodic GnRH secretion in rats and possibly sheep.

Many of the studies described here also examined the negative feedback actions of E_2, with the conclusion that complete or anterior knife cuts had no effect on the ability of E_2 to inhibit LH secretion in rats,[181] sheep,[161] and monkeys.[162] They also had no obvious effect on the negative feedback actions of progesterone in rats[182] and sheep.[183] Thus, both these steroids most likely exert their primary negative feedback actions within the MBH–pituitary unit. As noted in this chapter, these observations do not exclude additional sites of E_2 negative feedback outside the MBH, as has been observed in rats[184] and sheep,[185] but just illustrate that these sites are not essential for the normal feedback control of tonic GnRH secretion. Because the primary action of progesterone in most species is to inhibit pulse frequency, it has been inferred that progesterone must act in the MBH, and there is substantial evidence for this in sheep (see the section Neural Circuits Mediating the Negative Feedback Actions of Ovarian Steroids). In contrast, there has been considerable controversy about whether E_2 negative feedback occurs in the MBH or pituitary. This question has been clearly answered in sheep: during the estrous cycle, E_2 inhibits GnRH pulse amplitude (Figure 33.6),[186] probably by actions in the ARC,[185] in addition to direct inhibitory effects at the pituitary. There is also strong evidence in rabbits that E_2 inhibits both GnRH secretion and the pituitary response to GnRH,[76] and it is interesting to note that while E_2 inhibits GnRH pulse amplitude in both sheep and rabbits, it increases pulse frequency in sheep but decreases frequency in rabbits (Figure 33.6). In contrast to rabbits, current data in ferrets indicate that this steroid does not inhibit GnRH secretion.[191] Thus the negative feedback action of E_2 probably occurs at the pituitary in ferrets, although E_2-induced decreases in response to GnRH have been observed in some studies,[192] but not others.[193]

No clear conclusions can be drawn from studies of E_2 negative feedback in monkeys and rats. Early work in rhesus monkeys demonstrated that E_2 completely suppresses

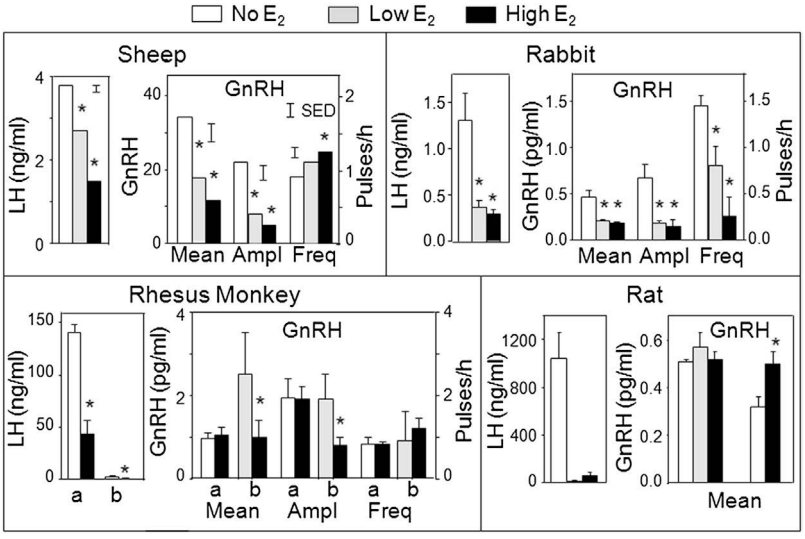

FIGURE 33.6 Negative feedback actions of E_2 on GnRH secretion measured in hypophysial portal blood in sheep,[186] and push–pull perfusions of the median eminence in rabbits,[76] monkeys,[187,188] and rats.[189,190] Mean (±SE) LH (in peripheral samples) and GnRH concentrations and GnRH pulse amplitude and frequency in sheep, rabbits, and monkeys are shown, but GnRH pulse parameters are not available in rats. Variabilities in data from sheep (SED) are presented as the standard error of mean of difference among groups from analysis of variance. Data from two different studies (labeled a[187] and b[188]) in rhesus monkeys are presented to illustrate variability in the effects of E_2. Data from rats compare mean GnRH values at 1300 h from OVX and intact ("low E" on diestrus and "high E" on proestrus) animals (bars on left)[189] and from OVX + E rats (bars on right).[190] Note that for data in sheep, mean GnRH is pg/10 min, while GnRH pulse amplitude is pg/pulse. *$P < 0.05$ versus no E group.

LH pulses maintained in MBH-lesioned animals by exogenous GnRH,[138] but these results do not preclude actions at both the MBH and pituitary. Local administration of E_2 within the MBH decreased gonadotropin secretion,[194–196] but high doses of estradiol benzoate given peripherally did not inhibit GnRH concentrations in cerebrospinal fluid[197] or in samples collected by push–pull perfusion of the median eminence[187] at a time when LH concentrations are clearly suppressed in OVX monkeys (Figure 33.6, study a). In contrast, a similar treatment inhibited GnRH concentrations by about 50% in samples collected from the median eminence of OVX monkeys pretreated with an E_2 implant that produced follicular-phase concentrations of this steroid (Figure 33.6, study b).[188] It should also be noted that all three of these studies used acute injections of estradiol benzoate that produced supraphysiological levels of E_2, so the relevance of the data to the normal negative feedback actions of E_2 is unclear.

The story in the rat is even more perplexing. Studies examining the chronic effects of peripheral implants[131] or intrahypothalamic microimplants[198] of E_2 have demonstrated a suppression of tonic LH secretion and a simultaneous increase in response of the pituitary to exogenous GnRH. The inescapable conclusion of these observations appears to be that these E_2 treatments are inhibiting GnRH secretion. However, when endogenous GnRH is measured in portal blood of rats, inconsistent effects of OVX are observed.[199,200] Furthermore, GnRH concentrations in push–pull perfusions of the median eminence of unanesthetized OVX rats are the same as, or lower than, those in ovary-intact rats (Figure 33.6),[189] and estradiol benzoate increased GnRH levels in morning samples collected by push–pull perfusion of OVX rats (Figure 33.6).[190] Thus, there is strong indirect evidence in the rat that E_2 inhibits GnRH secretion, but direct measurements of GnRH do not support such an action. Recent evidence that E_2 negative feedback occurs via ARC kisspeptin neurons in rats[3] (discussed further here) clearly favors the former conclusion, but no clear explanation for the lack of increased GnRH release in response to OVX has been advanced.

Neural Circuits Mediating the Negative Feedback Actions of Ovarian Steroids

There is now considerable evidence in a number of species that the negative feedback actions of progesterone occur via EOPs. Based on the effects of naloxone and similar EOP receptor antagonists, it is now clear that EOPs hold LH secretion in check during the luteal phase in a number of species. This was first reported in women[201] and has since been demonstrated in monkeys,[1] sheep,[2] and pigs,[202] and during pregnancy in rats.[203] Because the most consistent effect of naloxone is to increase LH pulse frequency in these studies, it was proposed that EOPs mediate progesterone negative feedback in these species. This hypothesis is supported by a number of reports that EOP antagonists have no effect in postmenopausal or oophorectomized women[1] or in long-term OVX sheep[2] or pigs,[202] but increase LH secretion when exogenous progesterone is given to women[204,205] or sheep.[2] In rats, EOP inhibition of LH may occur in the absence of progesterone because some studies reported that naloxone induced small increases in LH pulse amplitude in OVX rats.[206] However, there are also reports that naloxone had no effect in OVX rats,[207–209] and the effects that have been observed are less dramatic than those observed during pregnancy.[203] In contrast to these spontaneous ovulators, naloxone increases LH secretion in OVX rabbits[210] and ferrets,[211] but not in ovary-intact rabbits or estrogen-treated OVX ferrets, although the former studies were done when progesterone was low or absent. There is also one report that naloxone increases LH pulse amplitude in OVX guinea pigs,[212] but the effects of this antagonist have not been examined in ovary-intact guinea pigs. In summary, there is a consensus that EOPs mediate progesterone negative feedback during the luteal phase in those species in which this hypothesis has been tested. It is less clear which EOP is involved, but there is strong evidence that dynorphin plays this role in sheep[2] and some evidence that this is also the case during pregnancy in rats.[213]

While EOPs appear to mediate progesterone negative feedback in many species, their role in the negative feedback action of E_2 appears to be species specific. Thus naloxone increases LH secretion during the late follicular phase of the human menstrual cycle[1] and in oophorectomized women given exogenous estrogen,[205,214] but has no effect during the follicular phase of the monkey menstrual cycle.[215,216] There is one report that naloxone increases the frequency of LH pulses and MUA in OVX+E (ovariectomized E_2-treated) monkeys, but these authors concluded that this effect was not physiologically relevant because of the lack of effects of naloxone in follicular-phase animals.[217] While there is some evidence that EOPs mediate E_2 negative feedback on LH pulse amplitude in sheep, naloxone had equivalent stimulatory effects on GnRH pulse amplitude in OVX and OVX+E ewes.[2] In rats, naloxone increases LH secretion on all days of the estrous cycle[218] and restores episodic LH release in estrogen-treated OVX rats.[206,219] However, this EOP antagonist has equivalent stimulatory effects on GnRH secretion in ovary-intact and OVX rats, indicating that EOPs are unlikely to mediate E_2 negative feedback in this species.[220]

More recent work has focused on the possible role of ARC kisspeptin neurons in mediating the negative feedback actions of E_2.[156,221] These ARC neurons also contain neurokinin B (NKB) and dynorphin and thus are referred to as KNDy cells.[177] The evidence in support of this hypothesis comes largely from reports that E_2 inhibits kisspeptin expression in this region,[155] an effect that has been consistently observed in rats and mice,[3] hamsters,[222] musk shrews,[223] sheep,[2] monkeys,[1] and humans.[224] Since kisspeptin stimulates GnRH release, these inhibitory effects of

E_2 could mediate its negative feedback actions on GnRH. In rats, ablation of the ARC kisspeptin neurons with saporin conjugated to an NK3R agonist prevented the increase in LH concentrations following OVX, but some inhibitory effects of E_2 were still evident.[225] These authors thus concluded that ARC kisspeptin neurons play a major, but not exclusive, role in E_2 negative feedback. However other ARC neurons containing NK3R would also be lesioned by the techniques used and this proposed role for ARC kisspeptin neurons in rodents remains controversial.[3] Finally, it is interesting to note that, at least in sheep, the same population of KNDy neurons may mediate the negative feedback actions of progesterone and estradiol, but do so via different transmitters (dynorphin and kisspeptin, respectively).

Hypothalamic Control of the Gonadotropin Surge

Not surprisingly, given its central role in ovarian function, the mechanisms responsible for the preovulatory surge have been studied extensively, and consequently are better understood than those controlling tonic GnRH secretion. These mechanisms, including species variations in them, have been the focus of several recent reviews,[31,152,155,226] so this discussion will be more of a broad overview of the similarities and differences among species, again focused largely on rodents, sheep, and primates. The most obvious commonality across all these species is the stimulatory effects of E_2 on the pituitary response to GnRH (Figure 33.5). The temporal correlation between these stimulatory effects of E_2 at the pituitary and the time required for the estrogen-induced LH surge argues that these effects play an important role in the positive feedback actions of E_2. It should also be noted that the self-priming actions of GnRH on gonadotropes is estrogen dependent,[124,137] so this can be considered another aspect of E_2 positive feedback at the pituitary.

It is also clear that, in all species in which it has been measured, a GnRH surge accompanies the preovulatory LH surge (Figure 33.7). The strongest case for this is the ewe, in which GnRH can be monitored in hypophysial portal blood collected from unanesthetized animals.[229] GnRH measurements in rats,[189] rabbits,[227] and monkeys[228] have been done using push–pull perfusion of the median eminence. This is a technically challenging procedure, so it is not surprising that there is more variability associated with these estimates. Nevertheless, a significant increase in GnRH secretion coincident with the preovulatory surge has been identified in all four species. A second common feature observed across species, with one notable exception, is a dramatic increase in Fos expression (commonly used as an index of neural activity)

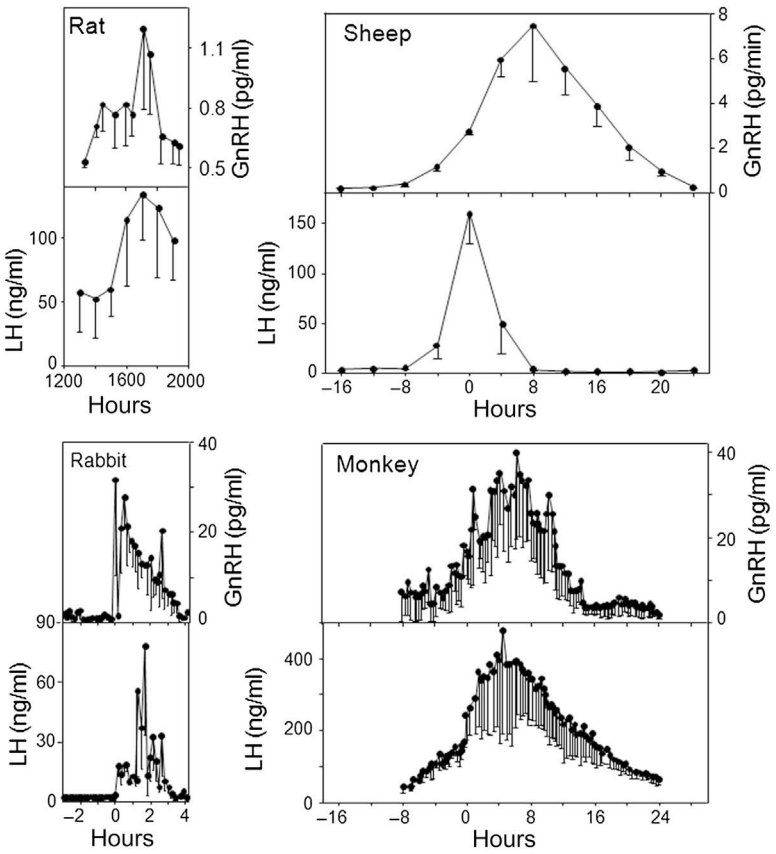

FIGURE 33.7 Endogenous preovulatory LH (in peripheral samples) and GnRH surges measured in hypophysial portal blood in sheep,[2] and push–pull perfusions of the median eminence in rabbits,[227] monkeys,[228] and rats.[189] Note that widths of plots have been adjusted to illustrate differences in the duration of LH and GnRH surges across species. *Redrawn with permission from the Endocrine Society.*

in GnRH neurons at the time of the preovulatory surge. This occurs in mice,[230] rats,[231] hamsters,[232,233] sheep,[234] and reflex ovulators such as the rabbit[235] and ferret,[236,237] with Fos seen throughout the anatomical distribution of GnRH cell bodies, including the MBH in those species in which GnRH neurons are found in this area (sheep, rabbits, and ferrets). The one exception to this pattern is the rhesus monkey,[238] which may relate to the important sites of E_2 positive feedback in this species (see the section Hypothalamic Areas Necessary for the Preovulatory Surge).

Hypothalamic Areas Necessary for the Preovulatory Surge

In contrast to the similarities of E_2 positive feedback at the pituitary among these species, there are significant species differences in the hypothalamic mechanisms underlying the activation of GnRH neurons during the preovulatory surge. The most obvious of these differences is between spontaneous and reflex ovulators. In the latter, which includes a large group of mammals,[6,7] stimulatory input from coitus to GnRH neurons is conveyed by numerous pathways. Thus in rabbits, complete de-afferentation of the hypothalamus is needed to block copulation-induced ovulation.[239] However, there are major differences in neural areas required for the surge even among spontaneous ovulators. The most dramatic of these is between rats and monkeys: in rats, knife cuts (either complete or partial) between the POA and MBH completely block the estrogen-induced LH surge[240] and ovulation (Figure 33.3),[35–37] while in monkeys similar cuts have no effect on menstrual cycles or the ability of estrogen to induce an LH surge.[162] In sheep[160,161] and guinea pigs,[163] the effects of these knife cuts are intermediate between these two extremes in that they do not completely block the estrogen-induced LH surge, but suppress its amplitude (Figure 33.8).

Differences in hypothalamic areas participating in the GnRH surge reflect underlying differences in the sites and mechanisms of E_2 positive feedback among these species. These have been best characterized in the rat,[3,31,152,155,226] in which a set of neurons in a region known as the rostral periventricular area of the third ventricle (RP3V),[226] which includes the anteroventral periventricular area (AVPV), mediate E_2 positive feedback. These neurons may receive neural afferents from the superchiasmatic nucleus (SCN) that gate the GnRH surge to a specific time of day[31]; if E_2 concentrations are elevated, this signal stimulates RP3V neurons, which in turn act on GnRH cell bodies in the POA[231] to trigger the preovulatory GnRH surge.[189] In sheep, there is strong evidence that E_2 acts in the MBH, and more specifically within the ventromedial nucleus (VMH)–ARC region, to induce the GnRH surge.[185] These systems, in turn, activate GnRH neurons in both rostral (POA and anterior hypothalamic area) and caudal (MBH) regions,[234] resulting in a prolonged GnRH surge,[229] which reflects a progressive change from episodic to non-episodic GnRH secretion.[241] In monkeys and humans it is clear that the changes in hypothalamic GnRH release are not required for the estrogen-induced LH surge[128,242] and ovulation,[242,243] because these can be produced in lesioned monkeys and women with Kallman syndrome (who have no GnRH neurons) by treatment with invariant episodic injections of GnRH. However, as reviewed recently,[152] there is also considerable evidence for a preovulatory GnRH surge in monkeys (Figure 33.7), although they are the one species studied in which Fos expression does not increase at the time of the surge.[238] The data in women are more equivocal: indirect indices of GnRH release suggest there is no increase at the time of the LH surge,[244,245] but the preovulatory LH surge is consistently blocked with GnRH receptor antagonists,[246,247] an effect that is unlikely to be due to disruption of the effects of GnRH pulses.[248] In reflex ovulators, it has also been difficult to identify stimulatory effects of E_2 on GnRH release because estrous behavior, which is a prerequisite for coitus, only occurs in E_2-treated females.[6] It is thus not possible to compare the effects of coitus in OVX and OVX+E animals, but there is strong evidence that E_2 is required for the stimulatory actions of NE (see the section Neural Circuits Responsible for the Control of the GnRH Surge by Ovarian Steroids) during the coitus-induced surge in rabbits.[249]

Neural Circuits Responsible for the Control of the GnRH Surge by Ovarian Steroids

The two neural systems implicated in the induction of GnRH surge across several species are: (1) NE, one of the oldest identified; and (2) kisspeptin, one of the newest

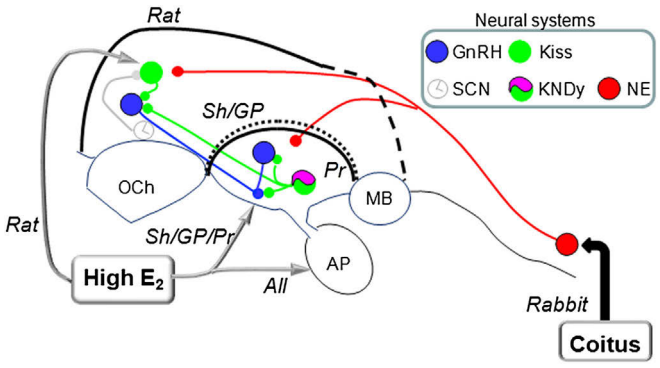

FIGURE 33.8 Schematic of parasagittal section of the hypothalamo–hypophysial unit illustrating the anatomical areas and neural systems participating in the preovulatory GnRH surge and sites of E_2 positive feedback in various species. This figure is reproduced in color in the color plate section. All structures are required in rabbits; in other species, solid lines depict knife cuts that had no effect on the surge. Dotted lines are cuts that only decreased the amplitude of the LH surge, and the dashed line illustrates that NE input is normally required for the surge in rats but can be compensated for under some circumstances. See the text for more details. AP: anterior pituitary; GP: guinea pig; MB: mammillary body; OCh: optic chiasm; Pr: primate; Sh: sheep.

entries into this arena. NE (along with acetylcholine) was initially identified as playing a role in the preovulatory LH surge by classic work in the late 1940s demonstrating that the α-adrenergic receptor antagonist, dibenamine, blocked ovulation in both rabbits[32] and rats[27] (Figure 33.2). Since then, abundant data have accumulated supporting this hypothesis in several species. As reviewed elsewhere,[6,7] the strongest case for a pivotal role for NE comes from work in the rabbit and includes evidence that coitus induces NE release in the median eminence just before GnRH secretion increases (Figure 33.9).[227] This temporal correlation reflects a stimulatory effect of NE on GnRH secretion because: (1) exogenous NE induces LH[250] and GnRH[251] secretion in intact and OVX+E, but not OVX, does[249]; and (2) an α_1-adrenergic antagonist blocks the coitus-induced increase in GnRH secretion.[252] To put this system into its physiological context: tactile stimuli from the vagina and cervix initiate action potentials that travel up the spinal cord to stimulate brainstem NE neurons, probably via a cholinergic pathway.[253] The A1 (medulla oblongata), A2 (nucleus of the solitary tract), and A6 (locus coeruleus) NE cell groups all project to the hypothalamus and are activated by coitus.[253–255] Thus, NE release from their efferents stimulates GnRH release by actions at both GnRH terminals in the median eminence[227,256] and GnRH cell bodies throughout the POA and MBH.[235] There is some evidence that the A1 and A2 groups may stimulate the A6 group and that the latter helps prolong NE release in the hypothalamus.[7,253] Despite this speculation, the mechanisms by which a brief (1–3 min) coital act is transduced into release of NE, and thus GnRH, which lasts for 2–3 h, remain a mystery.

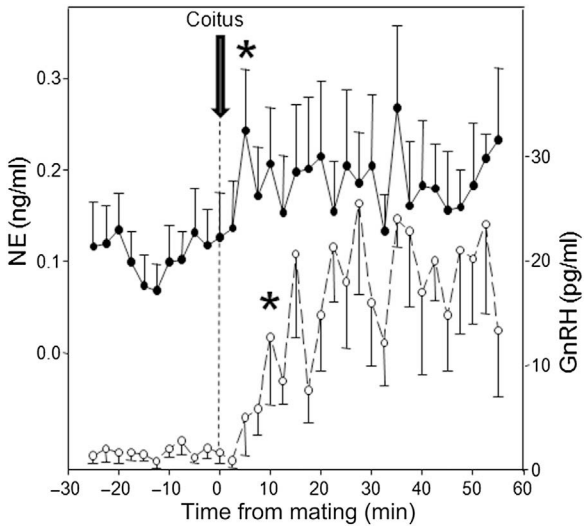

FIGURE 33.9 Effects of coitus (arrow) on mean (±SEM) concentrations of norepinephrine (NE; solid symbols and lines) and GnRH (open symbols and dashed line) measured in push–pull perfusions collected every 2.5 min from the rabbit median eminence ($n = 6$). Data averaged from plots of individual animals.[227] *First statistically significant increase in NE and GnRH over values before mating.

There are very limited data on the role of NE in other reflex ovulators, but there is considerable information on the role of this neurotransmitter in the LH surge in rats, sheep, and monkeys. The only other study in reflex ovulators reported that coitus increased Fos expression in A6 NE neurons but not in the A1 group in ferrets.[257] Interestingly, A2 NE neurons showed increased Fos in response to both coitus and male pheromones, suggesting that they are activated primarily by chemosensory cues. In spontaneous ovulators, NE release (estimated by microdialysis or turnover rate) increases in the POA and median eminence before and during the preovulatory LH surge in rats,[258,259] sheep,[260–262] and monkeys.[263] These species differ in the source of this NE input; both the A2[264–266] and A6[267–269] NE groups appear to be important in rats, whereas the A1[270,271] and A6[263] NE groups have been implicated in sheep and monkeys, respectively. The relative importance of NE to the normal surge also varies among these species (Figure 33.8), with α-adrenergic antagonists completely blocking the surge in rats,[27,259] but having no effect in primates.[41] The sheep appears to fall between these two extremes as phenoxybenzamine, a noradrenergic antagonist, delays or blocks the estrogen-induced surge in some, but not all, ewes, although the inhibition in some animals could reflect nonspecific effects because of the high dose used in this study.[272] It should also be noted that NE is not absolutely required for the LH surge even in rats because chronic disruption of all NE inputs from the brainstem does not block the ability of estrogen to induce surges in this species.[150] Thus it has been proposed that NE acts as a facilitatory agent in rats, increasing the responsiveness of GnRH neurons to other stimulatory inputs that drive the preovulatory GnRH surge.[264] In summary, NE plays an important stimulatory role in the preovulatory GnRH surge, but its importance varies from obligatory in rabbits, to facilitatory in rats (and possibly sheep), to redundant in monkeys.

There is a growing consensus that the rostral kisspeptin population plays a key role in the estrogen-induced LH surge. In rodents this population is concentrated in the RP3V, while in other mammals it is more scattered in the POA. There is very strong evidence that RP3V kisspeptin neurons mediate E_2 positive feedback in rodents,[3] and substantial evidence for a similar role for the POA kisspeptin population in sheep.[2] E_2 also increases kisspeptin expression in this region in guinea pigs[273] and musk shrews,[223] but apparently not in hamsters,[274] and Fos expression increases in these rostral kisspeptin neurons at the time of the surge in all of these species. It is interesting to note, in this regard, that pony mares are the one species in which rostral kisspeptin neurons have not been identified,[275] which may relate to the apparent absence of E_2 positive feedback in this species.[43]

While the proposed functional dichotomy in the roles of rostral (positive feedback) and ARC kisspeptin (negative feedback) populations in the actions of E_2 is largely

supported by data from rodents, it may not be applicable to other species. Specifically, in sheep[2] and guinea pigs[273] there is evidence that the ARC kisspeptin population is involved in both negative and positive feedback actions of E_2; the latter effects would be consistent with the sites of E_2 positive feedback in the MBH in these species (as discussed in this chapter). Whether this reflects subpopulations of ARC kisspeptin neurons dedicated to different effects of this steroid, or different responses of the same neurons to high and low E_2 concentrations, awaits further study, but a recent report in sheep favors the latter.[276] It is also important to note that this model is based on data from a limited number of species (primarily rats, mice, and sheep) so care must be taken in generalizing it to other mammals at this time. In particular, while it is clear that kisspeptin is critical for GnRH secretion in humans[277,278] and that kisspeptin expression in the ARC increases in postmenopausal women,[224] little is known about the physiological roles of kisspeptin in control of the menstrual cycle in monkeys[1] and nothing about its role in the control of the menstrual cycle in women.

While NE and kisspeptin have been implicated in the positive feedback actions of E_2, EOPs appear to mediate the ability of progesterone to block the GnRH surge in rats.[279] In contrast, EOPs play little, or no, role in mediating the ability of progesterone to block the LH surge in monkeys[280] or sheep.[2] These species differences may reflect differences in the role of EOPs in the normal LH surge. In rats, removal of endogenous EOP tone plays an important role in controlling the LH surge,[281] but EOP antagonists do not advance the LH surge in sheep[2] and there is no evidence that EOP withdrawal is important in primates.[280]

In comparing the neural areas and systems contributing to the preovulatory GnRH surge across species, an interesting continuum is evident (Figure 33.8). At one end are the reflex ovulators, exemplified by the rabbit, in which neural signals during coitus stimulate brainstem NE neurons whose input is absolutely essential for the GnRH surge. At the other end are nonhuman primates, in which no neural input (including NE) into the MBH is necessary for the LH surge, and humans in which there is no direct evidence that GnRH secretion increases at the time of the LH surge. Sheep and guinea pigs appear closer to primates than rabbits in this continuum, while rats appear closer to rabbits. In the former species, input from outside the MBH augments the amplitude of the GnRH surge, but is not necessary for it, and the role of NE is questionable. In the rat, both input from the POA and NE stimulation are normally required for the preovulatory LH surge, although compensatory mechanisms can replace NE input if it is chronically depleted. The similarity between rats and rabbits is further emphasized by evidence that rats can become reflex ovulators under some circumstances.[282,283] Interestingly, one of these examples involved disruption of inputs from the SCN to the POA,[283] so conceptually input from the biological clock in the SCN may have replaced the stimulus from coitus in the rat. The NE contribution to the GnRH surge in the rat may also reflect spillover of NE input to the hypothalamus, which is essential for the prolactin surge on proestrus[284] and mediates the coitus-induced increase in prolactin secretion responsible for pseudopregnancy in rats.[285]

Finally, it is interesting to note a similar continuum in the sexual differentiation of the GnRH surge in spontaneous ovulators. All species show a sexual dimorphism in the positive feedback actions of E_2, in that this steroid cannot induce an LH surge in adult males with normal testicular function, although the basis of this sexual dimorphism varies among species. In the rat, the absence of positive feedback is caused by the organizational actions of testosterone during development, which sexually differentiates the surge system so that it cannot respond to E_2 under any circumstances.[286] In contrast, E_2 induced a robust LH surge in male monkeys that were castrated as adults[287] and in hypogonadal men.[288] Moreover, some male monkeys showed apparently normal ovarian cycles when the testes were replaced with an ovary,[289] while similar procedures in rats resulted in constant estrus.[11] From a historical perspective, these observations reinforced knife cut studies that indicated that there were fundamental differences in neural mechanisms controlling the LH surge in primates and rats.[152] Sheep appear to fall between these two extremes because androgen exposure during development masculinizes the LH surge in females treated with an E_2 implant neonatally, but does not do so until later in life in ovary-intact females,[290] possibly because of masculinizing effects of prolonged exposure to E_2 after birth.[291]

PHYSIOLOGICAL DISRUPTIONS OF REPRODUCTIVE FUNCTION

The reproductive system differs from most other systems in the body in that it is not critical for survival of the individual. Thus it can be shut down for prolonged periods under physiological conditions if infertility is eventually advantageous for survival of the species (i.e., the individual survives to breed at a later date). The most common instance of this is the hiatus in reproductive function prior to puberty, but periods of physiological infertility in adults are more often the norm than the exception. Many species show annual periods of fertility and infertility that ensure the young are born into environmental conditions favorable for their survival. Reproductive function can also be suppressed by undernutrition, lactation, stress, and social interactions. This section will focus on comparative aspects of puberty and lactation and more briefly

consider other examples of reversible infertility because these are discussed in detail elsewhere in this book. Aging is also often associated with reproductive failure, but this topic is considered in Chapter 37, and the usefulness of a comparative approach to this topic is unclear because most species do not live long enough in their natural environment for this to be considered a physiological process. Even in humans, the occurrence of menopause is probably a fairly recent phenomenon because females probably did not live past middle age during most of the evolution of our species.

Puberty

In virtually all mammals, there is a hiatus between birth and onset of fertility, which allows the body to develop so it is physically and psychologically capable of rearing offspring. This is obviously important in females, who bear the energetic costs of pregnancy and lactation, but it is also important in males, who participate in rearing offspring in some species and must compete with mature males for access to females in many species. Because onset of fertility is a key event in propagation of the species, it should be expected that evolutionary pressures would result in differences among species and between sexes of the same species.[292] The latter is compounded by the duration of spermatogenesis; for example, although male rats are fertile (sperm in vas deferens) at a later age (55–60 days) than first ovulation in females (35–40 days), the first stages of sperm production must occur within the first week of life in males.

Considerations of the physiology of puberty usually begin with a discussion of its definition. On a superficial level, puberty can simply be considered the time when an individual becomes capable of producing viable offspring, but whether it is defined as a process or a single final event remains an open debate. The latter is often used in females because first ovulation, or first estrus, provides a single time point for the onset of fertility. In contrast, because of the duration of spermatogenesis, puberty in males is more often considered a developmental process. However, even in females it is clear that ovulation is preceded by a sequence of developmental events, in which different elements in this process (i.e., the ability of the follicle to respond to gonadotropins, and the positive feedback actions of E_2) mature at different times during the prepubertal period. This developmental process can be extended backward to consider important events during embryonic development (i.e., the development and migration of GnRH neurons, and the differentiation of gonadotropes), but for the purposes of this discussion, it is useful to focus on the final, rate-limiting steps leading to first ovulation and estrus in females, and initiation of spermatogenesis in males. Finally, it is important to point out that these definitional issues are not just of academic interest. For example, as discussed below, the relative weight one gives to specific developmental events depends on whether one considers menarche or first ovulation the onset of puberty in female primates.

If one focuses on the final step leading to fertility, a common feature emerges among those species that have been studied in detail: in both males and females, an increase in episodic secretion of GnRH is the rate-limiting step for onset of puberty.[293–295] In males, it is clear that such an increase will stimulate the secretion of FSH and LH necessary for testosterone and gamete production by the testes. In females, the situation is a bit more complex because the hypothalamus must be able to respond to the increasing concentrations of E_2 from the preovulatory follicle with a GnRH surge for ovulation to occur. However, the mechanisms responsible for the positive feedback of E_2 usually mature some time before first ovulation so they do not represent the rate-limiting event. One interesting exception to this generalization is monkeys and humans, in whom exogenous E_2 fails to induce an LH surge before the initial increases in GnRH that indirectly result in menarche.[295] However, there is strong evidence that this lack of E_2 positive feedback prior to menarche most likely reflects inadequate stores of LH in the pituitary due to the absence of endogenous GnRH pulses.[296] Moreover, the importance of episodic GnRH secretion to puberty in primates has been clearly demonstrated by the report that administration of exogenous GnRH pulses that mimic follicular-phase levels induced an increase in E_2 concentrations, an LH surge, and ovulation that resulted in a menstrual cycle in juvenile monkeys.[297] These data also support the role for GnRH pulses in providing sufficient pituitary gonadotropin for an LH surge because E_2 administration failed to induce an LH surge 30 days after the exogenous GnRH was discontinued in these prepubertal females. While there is thus general agreement that an increase in episodic GnRH secretion is a key step in initiation of puberty in many species, controversy remains as to the underlying neuroendocrine mechanisms in part because of species differences in them. These controversies can be broken down into three major issues: (1) the role of changes in response to steroid negative feedback; (2) the specific upstream neural (and glial) systems responsible for the increase in GnRH secretion; and (3) the relative roles of peripheral signals and developmental clocks in timing of puberty.

Historically, the first neuroendocrine mechanism for puberty proposed that LH release was suppressed in prepubertal animals by the negative feedback actions of low circulating gonadal steroids, and that at puberty a "resetting of the hypothalamic gonadostat" produced a decrease in sensitivity to this inhibition.[298] This change allowed GnRH secretion to increase until the negative

feedback loop was re-established at a lower set point so that adult concentrations of testosterone or estradiol were required to hold GnRH release in check. This proposal, which became known as the "gonadostat hypothesis," was initially formalized by Ramirez and McCann based on data in rats that doses of estrogen that were ineffective in adults were able to suppress the postcastration rise in gonadotropins in prepubertal animals. This hypothesis was soon extended to humans[299] and subsequently to several other species, including sheep,[300] cows,[301] guinea pigs,[302] and ferrets.[303,304] However, work in rhesus monkeys challenged this hypothesis because gonadotropins were undetectable throughout much of the prepubertal period in gonadectomized males and females, and then increased at about the age that puberty occurs in gonadally intact individuals.[295,305] Similar variations in gonadotropin secretion during development were also observed in agonadal children, so that it was proposed that "steroid-independent" inhibition of GnRH secretion was responsible for the prepubertal hiatus in primates.[295] The resulting debate on the relative importance of steroid-dependent and steroid-independent mechanisms to puberty lasted for over two decades, but has waned recently as workers focused on other issues. Moreover, close examination of the data suggests that both mechanisms contribute in many species, particularly in females (Figure 33.10). Perhaps the best example of this is the female rat, which is more sensitive to the negative feedback of E_2 prior to puberty than in adulthood. However, these inhibitory effects wane only after first ovulation,[306] and a modest steroid-independent increase in LH secretion evident as daily afternoon increments during the week preceding ovulation appears to be the ultimate signal for this seminal event.[307,308] While these data argue against the gonadostat hypothesis, it should be kept in mind that GnRH release is being held in check prior to puberty by E_2 negative feedback so that both steroid-dependent and steroid-independent systems contribute to the physiological concentrations of LH immediately before ovulation. Moreover, the decrease in steroid-dependent inhibition that occurs shortly after first ovulation most likely helps compensate for the negative feedback effects of the increased progesterone secretion occurring at this time,[306] and thus may well be critical for continued fertility. A similar temporal sequence of first steroid-independent and then steroid-dependent increases in GnRH occurs in primates,[295] with the former responsible for menarche, while the latter may be necessary for first ovulation.[309] Interestingly, the reverse sequence occurs in male hamsters, with steroid-dependent changes being responsible for the initial testicular growth up to 8 weeks of age, and a secondary steroid-independent increment apparently driving the sustained growth through 14 weeks.[310] Finally, even in species such as sheep and cows, where a decrease in E_2 negative feedback is the primary signal, steroid-independent increases in LH are evident in OVX animals at similar ages[300,301] and become rate limiting to puberty under conditions of low nutrition.[311,312] Thus, both mechanisms are evident in many species, with their

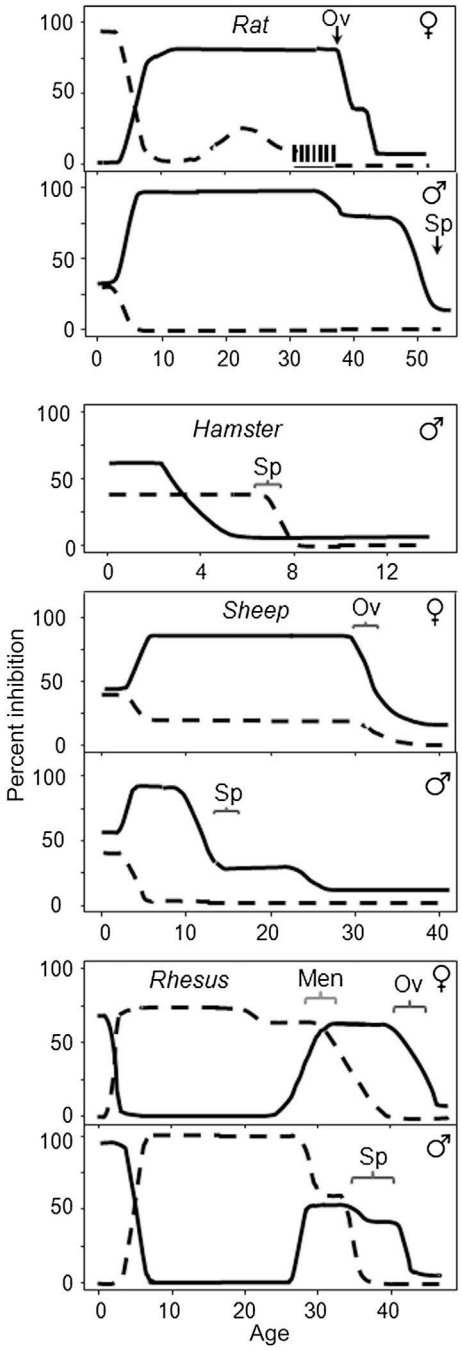

FIGURE 33.10 Schematic illustration of the time course of steroid-dependent (solid lines) and steroid-independent (dashed lines) mechanisms inhibiting LH secretion before and through puberty in female and male rats, sheep, and rhesus monkeys and male hamsters. Time scale is in days for rats, weeks for hamsters and sheep, and months for primates. Men: menarche; Ov: first ovulation; Sp: first appearance of mature sperm in reproductive tract.

relative importance changing with the phase of development and nutritional level. It is interesting to note that there can also be sex differences in the same species in the relative importance of these two mechanisms. For example, in contrast to females, puberty in male rats appears to be solely dependent on steroid-dependent mechanisms,[313] while the converse may be true in mice because LH concentrations increase following OVX, but not after orchidectomy, of prepubertal mice.[314]

There is less information available on the other two major issues under consideration: the neural systems underlying puberty and the ultimate signals that time these events. Until recently, there was no consensus on the important upstream neural systems, and there appeared to be major species differences.[293–295] For example, there is strong evidence that removal of an EOP inhibition of GnRH secretion contributes to puberty in female rats, but EOPs do not hold GnRH secretion in check in prepubertal monkeys, and although EOP receptor antagonists increase LH secretion in prepubertal sheep this EOP break does not decrease as the ewes go through puberty. Another dramatic example is NPY, which may play a stimulatory role during puberty in female rats and monkeys, but removal of its inhibitory actions may contribute to puberty in male monkeys. There is strong evidence for removal of a GABAergic inhibition and increase in stimulatory glutamatergic input in female rats and monkeys, but little direct support for this in other species. This story, of course, changed dramatically with the discovery of kisspeptin, which is now thought to be important for puberty in many species. There is now strong correlative and functional evidence that an increase in kisspeptin is the proximal stimulus driving GnRH secretion at puberty in humans, female monkeys, rats, and mice, and slightly weaker evidence in male monkeys and sheep.[156,293–295,315] In all these species, except mice, kisspeptin neurons in the ARC appear to be important for this process, and NKB produced by these neurons has more recently been implicated.[316–320] In mice, AVPV kisspeptin neurons may be as important as those in the ARC for puberty.[156] It has been argued that kisspeptin's role in puberty may not be regulatory in nature, but simply reflect its role as an essential component of the GnRH pulse generator.[321] In any case, identification of kisspeptin not only has been a major advance but also has moved the focus one step upstream of the GnRH neuron.

Much of the recent work in this field has focused on identifying the ultimate signals that determine at what age puberty occurs. Some of these, such as photoperiod in seasonally breeding animals,[294] are species specific but two potential mechanisms have been proposed to occur in most species[293–295]: (1) peripheral signals, indicating that critical maturational events have occurred and that sufficient energy reserves are available to reproduce; and/or (2) a developmental clock within the central nervous system that controls the timing of expression of a set of genes critical for puberty onset. For obvious reasons, work on the former has concentrated in general on metabolic (e.g., glucose) and related endocrine (e.g., insulin and IGF1) signals and specifically on leptin. In general (see the specific chapters on puberty[293–295] and Chapter 35 for detailed reviews), these studies demonstrated that insufficient concentrations of these signaling molecules delay puberty, but that increases in their concentrations do not immediately precede the onset of puberty. Thus, the consensus is that these indicators of metabolic well-being are permissive for, rather than determinant of, puberty onset.

There has been less work on a possible genetic clock in the central nervous system, although genetically programmed development is common in the reproductive axis since many of its components (e.g., GnRH neurons, gonadotropes, and cellular components of the gonads) develop to maturity before puberty occurs. Moreover, it has been estimated based on studies of dizygotic and monozygotic twins that 50–80% of the variability in age at menarche may be genetic in nature.[322] An interesting theoretical construct and some genes that may be components of this pubertal clock have recently been presented,[323] but human genetic analysis raises a cautionary note (see Chapter 32 for a more detailed discussion). Although linkage studies of rare genetic mutations producing infertility have produced major advances in our understanding of reproductive neuroendocrinology,[277,278,324,325] the genes identified by this method do not appear to contribute to the determination of age at menarche in the general population.[326] Furthermore, more systemic genome-wide association studies of age at menarche have to date only provided a few useful candidate genes for this theoretical puberty clock.[322] This may reflect methodological problems or the complex nature of the events leading up to menarche, which undoubtedly involve many genetic elements.[322] If the cause is methodological, recently developed techniques for high-throughput genomic and proteomic analysis[323] together with more powerful human genetic studies[322] are likely to provide significant progress over the next few years. Finally, it should be kept in mind that these two mechanisms are not mutually exclusive; an interaction of genetic signals, internal signals, and input from the external environment undoubtedly determine onset of puberty in most species.[292]

Lactation

Suppression of ovarian function during lactation occurs in many species, although there are some exceptions (e.g., the marmoset).[327] From an adaptive point of view, this period of infertility ensures that the mother

does not become pregnant during lactation because the latter is one of the most energetically demanding periods in her lifespan. However, postpartum ovulation and estrus occur in quite a few mammals (including rats, rabbits, ferrets, guinea pigs, horses, and kangaroos) so they are often pregnant and lactating at the same time. In some of these cases (e.g., guinea pigs and horses), gestation lasts much longer than lactation so that the latter is completed before the more energetically demanding later stages of pregnancy. In others (e.g., rats and kangaroos), lactation induces delayed implantation so that the later stages of pregnancy do not overlap with lactation. Nevertheless, adverse effects of combining lactation and pregnancy on both the developing fetuses and nursing offspring have been reported.[328] Because there was an excellent comparative consideration of this topic in the previous edition of this book,[327] in this section I will briefly compare the physiology of lactational infertility in a number of species and then describe recent developments in underlying mechanisms, based on data in rats. Readers interested in more in-depth consideration of this issue are referred to the previous edition of this book[327] and some excellent earlier[329,330] and contemporary[331] reviews.

Physiological studies on the mechanisms of lactational infertility have been largely limited to rats, pigs, sheep, cows, nonhuman primates, and humans. It is interesting to note, parenthetically, that rabbits have not been included in this group probably because does nurse their young for only about 3 min at the same time each day and show estrus within 24 h of pup removal.[332] They are thus more useful for studying suckling as a nonphotoperiodic zeitgeber for circadian rhythms[333] in both mothers[334] and pups,[335] than for its effects on fertility. In making comparisons across these other species, there are three potential confounding issues that should be kept in mind. First, rats ovulate immediately postpartum (in conjunction with their postpartum estrus) and, if not mated, become psuedopregnant because of the luteotropic actions of the elevated prolactin concentrations induced by the suckling stimulus.[327] Because any studies during normal lactation in rats are thus confounded by the elevated (compared to other species) circulating progesterone concentrations, many of these have been done in OVX animals. Second, in ewes (and some other seasonal breeders) lactational infertility usually overlaps with seasonal anestrus. Thus studies in this species have to be done in females that are bred out of season so that they lamb in the breeding season, and the significance of lactational anestrus to normal reproductive function is unclear. Third, comparisons of data from cows, which have been studied extensively because of the adverse economic consequences of lactational infertility,[336] with work in other species should keep in mind that dairy cows in particular have been bred to maximize milk yield. Thus the evolutionary (or selection) pressures in rats, sheep, and dairy cows may have been markedly different than those in other species, including primates.

Despite these caveats, some common patterns emerge when lactational infertility is examined across these species. First, in those species not showing postpartum estrus and ovulation, there is an initial period of infertility that is independent of lactation (Figure 33.11). This period, which occurs even if offspring are lost or removed, lasts from 2 weeks to 2 months depending on the species, and reflects the time needed for recovery of the hypothalamo–pituitary unit from the inhibitory effects of the high steroid concentrations of pregnancy and involution of the uterus. As this postpartum recovery progresses the inhibitory actions of lactation become dominant, but these effects can be divided into two phases. During approximately the first half of pregnancy the suckling stimulus itself, directly or indirectly (as discussed further below), inhibits reproductive function; while during the latter stages of lactation, as the suckling stimulus declines and the negative energy balance caused by the demands of the growing infants increases, the latter plays a more important role in suppressing fertility in the mother.

A second common feature is that ovarian inactivity reflects inadequate LH secretion, because FSH returns to normal follicular-phase concentrations shortly after parturition in most species.[327] In contrast, LH concentration

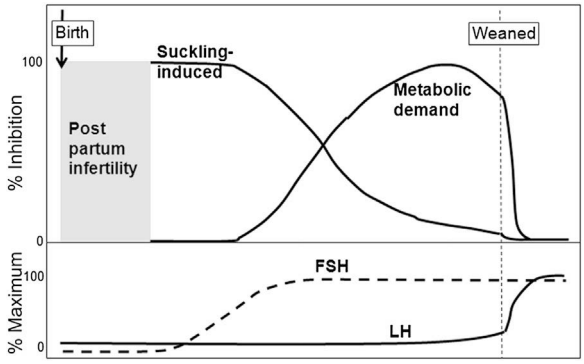

FIGURE 33.11 Schematic representation of systems inhibiting GnRH secretion (top panel) and the time course of FSH and LH concentrations (bottom panel) during lactation. In animals that do not have a postpartum ovulation, reproductive function is initially suppressed after parturition by lactation-independent effects of gestation (shaded bar). When the hypothalamus has recovered from the effects of gestation, GnRH secretion continues to be inhibited primarily by the effects of suckling, which may include both direct neural inhibition and the actions of elevated prolactin. As the offspring grow, the intensity of suckling decreases, but their metabolic demands increase so the latter becomes more important in suppressing GnRH secretion. In general, FSH returns to normal concentrations in the first half of lactation, so that episodic GnRH release and thus LH concentrations limit fertility. Weaning at any time after the postpartum period of infertility will result in a rapid increase in episodic GnRH secretion that soon results in ovulation.

and, more specifically, LH pulse frequency are suppressed throughout lactation and then increase rapidly shortly after weaning (Figure 33.11). As was the case with the prepubertal hiatus, both steroid-independent and steroid-dependent mechanisms contribute to the inhibition of GnRH pulse frequency in rats,[329,337] sheep,[338] cows,[339,340] and primates.[341,342] Nursing women are also more sensitive to the negative feedback actions of estrogen,[327] but steroid-independent inhibition has not been studied in women for obvious ethical reasons. In rats, there is strong evidence that steroid-independent mechanisms predominate in the first half of lactation, while increased response to E_2 negative feedback is critical during the latter stages.[337] This has not been systematically studied in other species, but the time course of LH concentrations in OVX lactating cows[340] and primates[341,342] is consistent with this possibility.

Species differences begin to emerge when the underlying mechanisms of this lactation-induced inhibition of GnRH secretion are examined. The suckling stimulus induces at least four important changes in hypothalamic function: (1) increased oxytocin secretion that is critical for milk ejection but probably plays no role in decreased GnRH release; (2) increased prolactin secretion that results from inhibition of ARC dopaminergic (DA) neural activity and is important for milk synthesis and secretion; (3) altered activity of orexigenic and anorexigenic neurons to increase maternal food intake; and (4) inhibition of GnRH secretion. The latter could reflect a direct effect of a neural signal initiated by suckling but could also be coupled to increased prolactin or changes in the neural circuitry controlling food intake. Hyperprolactinemia is well known to cause hypogonadism, so the possible role of prolactin has been examined in several species using a dopamine agonist (CB-154) to inhibit this hormone in nursing mothers. In OVX nursing rats, CB-154 partially restored LH concentrations in early lactation and completely overcame the inhibitory effects of sucking in the later stages.[343] Thus early in lactation prolactin significantly contributes to suckling-induced inhibition of GnRH and it may be the major inhibitor of GnRH during the second half of lactation in rats. In contrast, similar studies in sheep, cows, and pigs failed to provide any consistent evidence for an important role of hyperprolactinemia in lactational infertility.[327] Blockade of prolactin secretion increased the bursts of MUA coincident with LH pulses in some lactating monkeys, but the effects were inconsistent[344] and a review of the literature in humans concluded that prolactin plays only a minor role in lactational amenorrhea.[327]

Suckling produces a dramatic increase in food intake that correlates with increased activity of orexigenic neurons (NPY and agouti-related protein (AgRP)) and decreased activity of anorexigenic neurons (pro-opiomelanocortin (POMC) and cocaine-and-amphetamine responsive transcript (CART)) in the ARC.[331] Because NPY inhibits, and MSH and CART can stimulate, LH secretion, these changes could contribute to the suckling-induced suppression of GnRH secretion,[345] but recent data discussed later indicate this is unlikely in rats. Instead, these changes in orexigenic and anorexigenic neural activity probably assist the mother in compensating for the increased energy demands of lactation and most likely reflect both a direct neural signal and the abnormally low concentrations of leptin and insulin seen during lactation in rats[346] and cows.[347] In nursing women, leptin concentrations appear to be normal,[327] but increased insulin secretion (based on C-peptide) preceded the resumption of menses.[348] Thus, although hyperprolactinemia appears to be important in rats, neither the increased prolactin concentrations nor changes in the neural circuitry controlling food intake are critical for lactational infertility in other species. Interestingly, another possible mechanism arises from studies of nursing cows that have strongly implicated the formation of a maternal–calf bond, rather than the suckling stimulus per se, as critical for inhibition of GnRH secretion.[349] Whether this occurs in other species in which the mother can identify her own offspring, and the underlying neuroendocrine mechanisms, remain to be determined.

Virtually all work on neuroendocrine mechanisms suppressing GnRH during lactation since the previous edition of this book has been done in the rat, but it has led to an interesting model for the effects of suckling on hypothalamic neural circuits controlling food intake and the inhibition of GnRH secretion.[331] As noted above, suckling produces hyperphagia and there is a corresponding shift in the ARC circuitry critical for metabolic homeostasis (increased NPY and AgRP; decreased POMC and CART).[346,350–352] These ARC circuits are well known to stimulate downstream orexogenic neurons (orexin and MCH) in the lateral hypothalamic area (LHA) and inhibit anorexogenic CRH neurons in the PVN to increase food intake, changes that are also seen in lactation.[330] In addition, suckling induces NPY expression in the dorsomedial hypothalamus (DMH) neurons[352] that also project to the PVN[353] and have been implicated in suckling-induced hyperphagia.[354] The increased NPY in the DMH is partially due to the elevated prolactin concentrations because CB-154 suppressed NPY expression in the DMH, but not to levels seen in nonlactating controls.[355] All these appetite-regulating neurons (in the ARC, LHA, and PVN) also project to GnRH cells in the rat and could therefore directly inhibit GnRH release.[331] Alternatively, suckling may act via kisspeptin because it inhibits both kisspeptin and NKB (but does not affect dynorphin) expression in KNDy neurons.[337,346,356,357] The effects of suckling on kisspeptin expression in the AVPV is less clear; there are reports that *Kiss1* mRNA levels either did not change[357] or decreased[356,357]

during lactation, but the number of kisspeptin-immunoreactivity (kisspeptin-ir) cells in the region increased in lactating rats.[356]

The relative importance of all these changes was clarified by the report that increasing leptin and/or insulin in lactating rats to concentrations seen in normal controls restored expression of ARC NPY, AgRP, and POMC to normal, but had no effect on ARC kisspeptin–NKB or DMH NPY expression.[346] Importantly, these treatments had no effect on the hyperphagia or low-LH concentrations induced by lactation. Thus the ARC metabolic circuitry is likely responding to the increased metabolic demands of lactation, but is not critical for the behavioral and reproductive effects of this condition. Since these ARC systems drive neural activity in the LHA and PVN, it is likely that levels of orexin, MCH, and CRH were also partially or completely normalized, although this has yet to be demonstrated. Instead, the current model proposes that suckling stimulus, conveyed in part by neurons in the lateral parabrachial nucleus and ventrolateral medulla,[358,359] acts to directly stimulate DMH NPY and inhibit ARC KNDy and DA neurons (Figure 33.12). The increased prolactin concentrations, resulting from the latter, likely complements these effects because prolactin increases DMH NPY[355] and inhibits ARC kisspeptin expression,[360] and both these cell populations contain prolactin receptors.[355,361] The increase in DMH NPY then stimulates food intake, and the decreased release of kisspeptin inhibits GnRH secretion. NPY projections from the DMH may also inhibit GnRH secretion, but there is no clear evidence for such an action at this time. Thus, this model proposes that the suckling stimulus and prolactin act in concert to directly and indirectly inhibit kisspeptin, and thus GnRH, release. It is also important to keep in mind that the effects of leptin and/or insulin replacement indicate that the ARC metabolic circuitry is not required for inhibition of GnRH, but do not exclude the possibility that they contribute to this suppression. Given the redundancy in both reproductive and metabolic neuroendocrinology, it seems prudent to suggest that increased input of orexogenic (NPY, AgRP, orexin, and MCH) and decreased input of anorexigenic (MSH and CART) to GnRH neurons work together with the decreased kisspeptin drive to maintain infertility during lactation. Finally, while this is a very attractive model, its applicability to other species that do not show a postpartum ovulation and in which prolactin probably plays a minor role in suppression of reproduction is completely unknown.

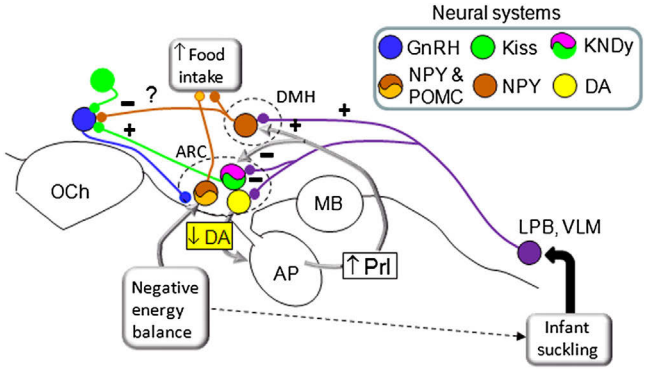

FIGURE 33.12 Neural systems stimulating food intake and inhibiting GnRH secretion during lactation in the rat. This figure is reproduced in color in the color plate section. The suckling stimulus is transmitted to the hypothalamus via neurons in the lateral parabrachial nucleus (LPB) and ventrolateral medulla (VLM), inhibits DA release from tuberoinfundibular dopaminergic (TIDA) neurons and kisspeptin release from KNDy neurons, and stimulates NPY neural activity in the DMH. The fall in DA allows prolactin secretion to increase, which further inhibits KNDy kisspeptin and stimulates NPY from the DMH. The latter increases food intake, while the former inhibits GnRH secretion. At the same time, the negative energy balance in the mother induced by the demands of the offspring acts via the metabolic homeostatic circuitry in the ARC (increased NPY–AgRP and decreased POMC–CART), which also acts to stimulate food intake and may inhibit GnRH secretion. Note that this circuitry is depicted by a single neural element (NPY and POMC) for simplicity. Similarly, orexogenic neurons in the LHA and anorexogenic CRH neurons in the PVN are not illustrated.

Other Instances of Physiological Suppression of Reproduction

As noted earlier, reproduction is also inhibited seasonally and by undernutrition and stress in many species, and is often modified by social interactions and pheromonal systems. Since most of these topics are considered in detail elsewhere in this book, this discussion of them here will be fairly brief with an emphasis on comparative aspects.

Seasonal Breeding

Most species living in temperate zones, and many of those in the equatorial region, have evolved mechanisms that ensure that their young are born at the time of year best suited for their survival. Given its obvious evolutionary significance, it is not surprising that a wide variety of reproductive strategies have been adopted to limit the timing of births, but two of these are fairly common and relevant here: (1) limiting fertility of the adult to a specific time of year; and (2) prolonging pregnancy by inducing a period of embryonic diapause. It is also useful to divide seasonality based on the two major environmental cues a species uses to time reproduction: (1) food availability; or (2) photoperiod. Small mammals with short pregnancies often couple their fertility to food availability because an increase in food will almost always last from fertilization through lactation.[362] There is evidence that some species use a specific plant compound to time reproduction,[363] but in most cases fertility is regulated by metabolic compounds (e.g., glucose availability) and

related hormones (e.g., leptin and insulin), presumably by the same neuroendocrine pathways by which these factors control reproduction in rats and mice. Because these pathways are described in detail in Chapter 35. I will focus the rest of this discussion on photoperiodic control of reproduction.

Animals whose pregnancy lasts several months routinely use photoperiod (hours of light/day) as the environmental cue because it remains exactly the same from year to year, and is thus a reliable predictor of when spring will occur. Moreover, in all mammals (from marsupials on up) that use photoperiod, the pineal hormone, melatonin, is the internal signal that times critical reproductive events. Melatonin is only secreted at night, so the duration of melatonin secretion is a hormonal analog of the external photoperiod. The duration of elevated melatonin then alters secretion of prolactin or GnRH to control embryonic diapause or fertility, respectively. Embryonic diapause usually occurs at the blastocyst stage of development and is characterized by cell cycle arrest.[364,365] The blastocyst then remains in suspended animation until the appropriate signal reinitiates mitosis and implantation soon follows. In species in which photoperiod controls this reinitiation process, a change in prolactin secretion, acting via ovarian hormones, is the primary trigger. Interestingly in all mammals studied to date, long photoperiods (i.e., short-duration melatonin) stimulate prolactin secretion, while short photoperiods inhibit secretion. Thus species have evolved different downstream actions of prolactin depending on whether implantation occurs on long or short days. For example, in mink diapause termination takes place on long days due to a spike in ovarian secretion of progesterone (and an unidentified protein hormone) in response to increasing prolactin.[365,366] The converse occurs in the tammar wallaby; elevated prolactin inhibits progesterone secretion from the ovary so the fall in prolactin produced by short days stimulates progesterone secretion, which terminates diapause so implantation occurs.[364]

In contrast to prolactin, the effects of photoperiod on GnRH secretion depend on whether an animal is a short-day or long-day breeder. Long-day breeders have short pregnancies so GnRH release is high, and breeding occurs during spring and summer, while short-day breeders have pregnancies that last about 5–6 months so elevated GnRH and breeding occur in the fall and early winter. These effects of photoperiod on GnRH release are further complicated by internal timing mechanisms that allow animals to anticipate a coming season (see Chapter 34). In the few mammals that have been extensively investigated (primarily sheep and Siberian and Syrian hamsters), inhibitory photoperiods (regardless of whether they are long or short days) suppress GnRH secretion by both steroid-dependent and steroid-independent systems, although the relative importance of each depends on the species.[367,368] However, the actual neural systems involved vary among these three species. Changes in kisspeptin appear to be important in sheep and Syrian hamsters, but not in Siberian hamsters.[369] Increased RFRP3 (RFamide-related peptide 3) in the DMH has been implicated in stimulating GnRH secretion in both hamster species,[369] but may have inhibitory actions in sheep.[2] Finally, there is strong evidence in sheep that activation of an inhibitory dopaminergic system (A15) that suppresses kisspeptin secretion is critical for seasonal infertility in anestrus,[370] but there is no evidence for an analogous system in hamsters. Thus, each of these seasonal breeders appears to have evolved different hypothalamic mechanisms to control GnRH secretion.

Despite the marked differences in the neural mechanisms by which melatonin controls fertility and embryonic diapause, recent work has uncovered possible convergence in the initial steps by which it acts. Specifically, there is now increasing evidence that long-day melatonin patterns act in the pars tuberalis of sheep and hamsters to stimulate prolactin secretion and alter GnRH release. The pars tuberalis contains the highest concentrations of melatonin receptors in the body, and melatonin is thought to act here to stimulate a prolactin-releasing factor (or "tuberalin"), which may be substance P, that stimulates prolactin secretion from lactotrophs during long days. However, it is unclear at this time if this mechanism applies to the actions of melatonin that control embryonic diapause. Melatonin has also been proposed to control GnRH secretion in hamsters and sheep by stimulating TSH secretion from thyrotrope-type cells in the pars tuberalis. This TSH then diffuses into nearby tissue to alter expression of enzymes that de-iodinate T_4 and produce a local increase in the biologically active T_3 during long days, which is responsible for altering GnRH secretion (to stimulate in long-day, and inhibit in short-day, breeders). The mechanisms by which T_3 produces these effects and whether this model also applies to the control of GnRH by short-day melatonin patterns remain to be determined. Readers interested in a more detailed discussion of melatonin secretion and actions, and the neural pathways controlling GnRH in seasonal breeders, are referred to the Chapter 34.

Stressors

It is clear that many different types of stressors inhibit reproductive function (see Chapter 36), although stimulatory effects are sometime observed.[371] Inhibition of the estrogen-induced LH surge has been observed with some, but not other, stressors, whereas all stressors inhibit tonic LH secretion.[371,372] Because a decrease in episodic LH secretion will indirectly block ovulation (by preventing the necessary increase in E_2 secretion), most workers have focused on stress-induced inhibition of pulsatile LH

secretion. It appears that the inhibitory effects of stressors are usually mediated by central mechanisms rather than by actions of elevated glucocorticoids.[371,372] Thus acute treatments with glucocorticoids have little effect on episodic LH secretion in rats, monkeys, and humans. Cortisol does act at the pituitary to suppress LH pulse amplitude in ewes[373]; this action is not required for the inhibition of episodic LH secretion by endotoxin,[374] but may play an important role in the effects of psychosocial stress.[375,376] Moreover, cortisol clearly suppresses LH pulse frequency during the follicular phase of the ovine estrous cycle,[377] so sheep may be one species in which glucocorticoids have important inhibitory effects on GnRH secretion.

Although central systems mediate the inhibition of GnRH by stressors, a comparative approach to identifying common neuroendocrine mechanisms underlying this inhibition reveals many more differences than similarities. This lack of common mechanisms may be due in part to the different types of stressors used, the most common of which are immunological (endotoxin or cytokines), insulin-induced hypoglycemia, restraint in rats, and psychosocial stress in sheep and monkeys. Although these stressors all increase corticotrophin-releasing hormone (CRH), vasopressin (AVP), adrenocorticotropic hormone, and glucocorticoid secretion, they presumably activate different sensors that impinge on both the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–gonadal (HPG) axes via different pathways. For example, hypoglycemia is likely to activate glucose sensors in the hind brain[378] not affected by endotoxin, so it is not surprising that NE input from this region to CRH neurons in the PVN has been implicated in suppression of GnRH by insulin-induced decreases in blood glucose.[372] However, species differences are evident even if one focuses on a specific stressor. For example, there is strong evidence that CRH mediates the inhibitory actions of cytokines in monkeys, but not in rats.[371] However, based on the effects of naloxone, EOPs participate in suppression of LH secretion by a variety of stressors in rats, sheep, monkeys, and humans, the one exception being hypoglycemia in monkeys.[371] There are also significant individual variations in sensitivity to some stressors that can complicate identification of important systems.[379] Finally, as noted in the discussion on lactation, there are also undoubtedly many redundancies in the systems activated by a specific stressor, so workers are unlikely to find one system that is absolutely essential to suppression of GnRH secretion.

Despite the problems inherent in making species comparisons, some useful generalizations can be made on the neuroendocrine inhibition of GnRH secretion by stressors. Given their role in the HPA axis, considerable attention has focused on AVP and CRH. AVP has received less attention and the data are somewhat inconsistent, but it does appear to play some role in the inhibition of GnRH secretion by hypoglycemia and cytokines.[371,372] In contrast, CRH appears to be an important mediator of many stressors. There are, however, two important caveats to this general statement: (1) in ewes, CRH either stimulates[380] or has no effect[381] on LH secretion, so it is unlikely to be important for stressed-induced inhibition of GnRH, possibly because cortisol is more important in this species; and (2) in rats, many stressors act via CRH receptor 2 (CRHR2), not CRHR1, so that urocortins may also be important mediators.[382] Other useful concepts that have recently emerged are the importance of limbic CRH systems and the interaction of CRH with aminergic neurons in the brainstem. Given its role in emotional processing, it is not surprising that neurons containing CRH in various portions of the limbic system have been implicated in the inhibition of GnRH by stressors.[372] There is strong evidence that some of their inhibitory effects occur by activation of NE neurons in the locus coeruleus.[383] Reciprocal connections between CRH neurons in the PVN and serotonergic neurons in the raphe nucleus have also been implicated in the inhibition of menstrual cycles in monkeys by psychosocial stress.[379]

In comparing the inhibition of GnRH secretion by stressors with seasonal and lactational effects, it is interesting to note that while stressors are clearly inhibitory in OVX animals, they are generally more effective in the presence of E_2 and/or progesterone than in their absence.[384–387] These data have usually been interpreted as an action of ovarian steroids to increase the effects of CRH or other mediators of stress, but could equally be viewed as a stressor producing both steroid-independent and steroid-dependent inhibition of GnRH. Recent work in this area has focused on kisspeptin, with the only two papers reporting that endotoxin induced a decrease in *Kiss1* mRNA[388] or protein[389] expression in the ARC. The former observed similar effects of this and other stressors and CRH on *Kiss1* and *Kiss1r* mRNA expression in the ARC and POA. Exogenous kisspeptin did stimulate LH secretion in the presence of endotoxin, but the magnitude of the response was markedly reduced compared to that in controls.[389] Thus the functional significance of the stress-induced decrease in kisspeptin remains to be determined. Finally, as noted above, many effects of stressors in general, and CRH in particular, appear to be mediated by EOP.[371] These data led to the suggestion that some of these actions may be mediated by increased dynorphin release from ARC KNDy neurons.[372] This hypothesis has yet to be tested, but earlier reports that an antagonist to μ-EOP, but not κ-EOP, receptors blocked effects of some stressors argues against it.[390,391]

Nutritional Suppression of Reproduction

Reproductive function is usually suppressed whenever a female is in a condition of negative energy balance to help ensure she survives and can reproduce at a later date. This condition can occur during periods of

high demand such as lactation (see the section Lactation) and intense exercise regimens even though food intake is increased, but the more common cause is inadequate nutrition, particularly in animals living in nonequatorial regions where food scarcity in winter is the norm.[392] Smaller mammals are particularly susceptible to the adverse effects of limited food because they have little energy stored as fat and a high surface-to-volume ratio that dissipates heat rapidly. Thus they must have a higher metabolic rate to maintain normal body temperature and must eat more food per unit body weight than larger animals.[392] Similar logic indicates that growing animals are more susceptible to food restriction than adults, and this is evident by the marked effects of undernutrition on the timing of puberty. While males and females of the same species are generally subject to the same energetic demands, these are usually more likely to produce infertility in females than males. This sexual dimorphism reflects the higher and more long-term energy demands on the mother during both pregnancy and lactation[392]; in contrast, while males may expend considerable energy in competition with other males, this usually lasts for a relative short period and is less energy demanding than pregnancy and lactation.

The effects of nutrition are often evident through their interaction with other factors that control fertility. As already noted, inadequate nutrition will delay puberty, and signals of adequate nutrition such as leptin concentrations are often permissive for puberty onset. Another obvious example is lactation, when the negative energy balance produced by the offspring acts in synergy with the suckling stimulus to suppress GnRH secretion. Moreover, nutrition also plays an important role in seasonal breeding. As discussed earlier, in some species with short pregnancies, fertility is controlled directly by levels of nutrition, which leads to a seasonal pattern because of the seasonal nature of food availability. Even in species that rely on photoperiod as the proximate cue to time fertility, food availability is thought to be the ultimate factor that provided the evolutionary drive that determined when breeding occurs (i.e., it ensures that the young are born when food is plentiful, usually in the spring). Finally, the effects of undernutrition and stress are often confounded. As discussed above, one of the most commonly used experimental stressors is insulin-induced hypoglycemia, which may be inhibiting GnRH secretion via metabolic sensors, rather than stress-sensitive neurons. Conversely, food deprivation is undoubtedly a stressor under most circumstances, so the resulting suppression of fertility may reflect activation of a stress system. It is thus not surprising that there is some convergence of signals generated by food deprivation and stress onto the PVN.

Mammals rely on multiple signals to monitor food availability and energy reserves in order to control fertility, including vagal input from the GI track, and circulating concentrations of metabolic fuels and their associated hormones.[378] There is considerable evidence in rodents that input from the vagus nerve and neural sensors in the lining of the fourth ventricle that detect a fall in glucose or fatty acids converge on the A2 NE neurons (solitary tract nucleus) in the brainstem.[378,393–395] These NE neurons send projections to the PVN, where they stimulate activity of CRH-containing neurons that inhibit GnRH secretion.[396–398] The mechanisms by which leptin, insulin, and other metabolic hormones affect GnRH secretion are a very active area of research. As described in Chapter 35, these hormones likely act via the NPY–AgRP and POMC–CART circuitry in the ARC (and possibly their downstream targets in the LHA and PVN) to influence both food intake and GnRH secretion and leptin-sensitive neurons in the ventral premammillary nucleus to control GnRH release.[399]

Inadequate nutrition inhibits GnRH secretion via both steroid-independent and steroid-dependent mechanisms[400,401] and the A2 NE input to the PVN appears to primarily increase the negative feedback actions of E_2,[394] possibly via changes in expression of ERα.[402] Decreases in kisspeptin also appear to play a central role in mediating the effects of undernutrition, although the relative importance of kisspeptin neurons in the ARC and POA is still under debate (see Chapter 35). Although some ARC kisspeptin cells contain leptin receptors, recent work indicates that the effects of leptin on kisspeptin expression and reproductive function are most likely mediated by leptin actions on other neural systems.[399,403]

Psychosocial and Pheromonal Control of Reproduction

Successful reproductive function requires coordinated complex behavioral interactions between males and females, and there is considerable information on the hormonal control of these behaviors. Conversely, psychosocial interactions can also influence secretion of GnRH (Chapter 36). One clear example of this is the marked difference in testosterone concentrations between dominant and submissive males in a troop of monkeys,[404] although the low testosterone in the latter might be due to stress-induced suppression of GnRH secretion.[405] The best studied example of psychosocial control of reproduction involves communication via pheromones, although these chemical signals can be supplemented by visual and auditory signals under some circumstances.[406] Pheromonal input can be stimulatory or inhibitory; for example, adult male urine will advance puberty onset in rodents, while urine of adult females delays first ovulation.[406] In most cases, the effects of pheromones on reproduction occur via changes

in GnRH secretion, but the ability of novel males to interrupt pregnancy in mice is mediated by inhibition of the prolactin secretion (via increased DA) necessary for maintenance of pregnancy in these rodents.[407]

There have been two important developments in our understanding of how pheromones influence GnRH release in the last decade that relate to the initial and ultimate effects of these chemicals. Historically, based largely on lesion studies in rodents, the vomeronasal organ, rather than the main olfactory epithelia, was thought to be the site where pheromones that affect reproductive function are sensed.[406] A multisynaptic neural pathway originating in this structure that projects to the hypothalamus via the accessory olfactory bulb and medial amygdala conveys pheromonal information to GnRH neurons. However, elegant tract-tracing studies using transgenic mice clearly demonstrated that cells in the main olfactory epithelia, the main olfactory bulb, the olfactory cortex, and the cortical nucleus of the amygdala represent a multisynaptic pathway from the main olfactory epithelia to GnRH neurons.[408,409] Moreover, this olfactory system conveys important pheromonal information to GnRH neurons based on Fos expression and genetic lesions of the main olfactory epithelium[408,409]; Fos data also confirmed a role for the secondary olfactory system.[408] Similar lesion and Fos expression studies in sheep have also implicated the main olfactory system in the ability of male odors to stimulate episodic LH secretion in anestrous females.[410–412] Interestingly, in contrast to just male odors, exposure to male sheep increased LH pulse frequency via both main and accessory olfactory systems in ewes. Thus, the general consensus at this time is that pheromones act via both main and accessory olfactory systems to stimulate episodic GnRH secretion.

Information on the final steps in this pathway comes largely from work in sheep and goats examining the ability of males to stimulate episodic LH release (in sheep)[2,413] and the corresponding bursts in MUA recorded from the ARC (in goats).[166] A series of experiments in OVX goats have demonstrated that brief exposure to male odor induces a single burst of MUA within seconds, suggesting that pheromonal information has direct access (via one of the pathways described above) to the GnRH pulse generator.[166,414] This hypothesis is consistent with evidence that male pheromones stimulate LH pulses in both anestrous and luteal-phase ewes,[415] which indicates that this stimulation is likely independent of the negative feedback actions of ovarian steroids. It is also supported by the recent report that ARC kisspeptin neurons mediate the stimulatory effects of the male in anestrous ewes.[416] Thus it seems likely that, in sheep and goats, stimulatory pheromones exert their effects directly on the GnRH pulse generator in the ARC; whether this also occurs in other species is unclear at this time.

Summary

It is not surprising that different external factors use different neuronal systems to control fertility. However, despite this diversity some common features emerge from this review. First, many of these modulators of reproduction produce their primary effects by controlling episodic GnRH secretion. Thus LH, not FSH, secretion is usually rate limiting for initiation, or reinitiation, of fertility. Second, many of these systems inhibit GnRH secretion via both steroid-independent and steroid-dependent mechanisms (Figure 33.13) similar to those operative in prepubertal animals, although the relative importance of each varies. Whether these mechanisms represent completely independent neural systems, or the same system that functions at a low level in gonadectomized animals and is stimulated by gonadal steroids, remains to be determined. Third, changes in activity of the GnRH pulse generator are responsible for most, if not all, of the actions of the neuroendocrine systems activated by external factors. This is not surprising given the importance of episodic GnRH secretion to control of fertility, but it does identify key neural systems that can be the focus of future research. Finally, changes in kisspeptin have also been implicated as the penultimate neural system driving GnRH secretion. This may reflect the possible role of kisspeptin as the output signal from the GnRH pulse generator (Figure 33.13) or other actions of kisspeptin related to its potential role in the negative and positive feedback actions of ovarian steroids.

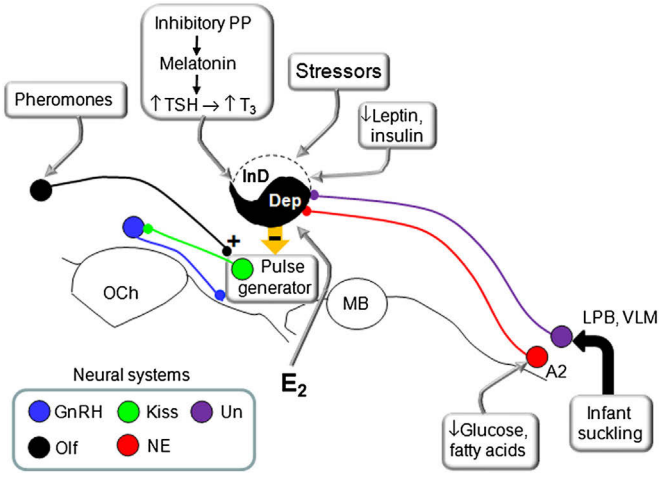

FIGURE 33.13 Schematic of parasagittal section of the hypothalamus illustrating mechanisms by which different external factors alter episodic GnRH secretion. This figure is reproduced in color in the color plate section. Note that neural inputs are conceptual and do not represent monosynaptic pathways. See the text for more details. Dep: steroid-dependent mechanisms; InD: steroid-independent mechanisms; Kiss: kisspeptin; NE: norepinephrine; Olf: olfactory epithelium; Un: unknown neurotransmitter.

CONCLUSION

From a historical perspective, it is clear that comparative studies of the neuroendocrine control of reproductive function played a key role in the development of the concept of tonic and surge secretion of LH. Comparative studies have also led to a better understanding of the multitude of neuroendocrine systems and pathways that can influence GnRH secretion, and some of the fundamental observations were initially made in species not commonly used for experimental work. One is particularly struck by the number of important observations first made in primates that laid the foundation for subsequent work in rats and mice. In addition to the identification of kisspeptin based on genetic studies in humans, these include the initial observation of episodic LH secretion (and the inference that this mode is due to intermittent GnRH release), and the identification of the importance of episodic GnRH secretion to normal LH and FSH secretion and of its electrophysiological correlate (bursts in MUA), all of which came from work in nonhuman primates. It is also important to keep in mind that from the perspective of a true comparative endocrinologist, the number of species in which detailed reproductive neuroendocrine control systems have been identified is small and not very diverse.

With the caveat that we are dealing with a relative small number of species, two general conclusions can be drawn from these species comparisons. First, there are marked species differences in the neural systems controlling the preovulatory LH surge, ranging from reflex ovulators that require an external signal to primates that apparently do not require any change in GnRH secretion for normal ovulation. In contrast, the endocrine signal (a sustained increase in E_2 concentrations) has been relatively conserved during evolution, because it serves to limit the LH surge to the time when a mature ovum is present in the follicle. Given this contrast, it is difficult to envision environmental pressures that led to the diversity of neural mechanisms responsible for the preovulatory GnRH surge. Perhaps as long as the estrogen signal was retained, there was little natural selection pressure to limit development of new neural systems. Second, in contrast to the diversity in neural mechanisms responsible for the LH surge, the neural systems driving episodic GnRH secretion appear to be fairly similar in those species examined to date. Moreover, the GnRH pulse generator is also the common final pathway for control of fertility by a wide variety of external factors (Figure 33.13). This may reflect the importance of episodic GnRH secretion, but not the GnRH surge, to fertility of *both* males and females, in contrast to the LH surge. This common role for the GnRH pulse generator in both sexes may also explain why it was targeted by evolutionary pressures for modification by the external environment.

This overview of the comparative aspects of reproductive neuroendocrinology highlights the importance of this approach to our understanding of basic mechanisms controlling GnRH secretion. Unfortunately, the prospects for future work in this field seem bleak. Over the last decade, the number of detailed reproductive neuroendocrine studies in nonrodent species has dwindled significantly. For example, in light of the historical impact of studies in the rabbit, it is remarkable that there is no information on kisspeptin expression in this species over a decade since the discovery of the reproductive actions of this peptide. Moreover, given the emphasis on genetic manipulations and the growing expense of nonhuman primate work, this trend is likely to accelerate. Thus important comparative issues may not be addressed. For example, although it is clear that both kisspeptin and NKB are critical for normal fertility, their physiological roles in the human, and other primates, menstrual cycle are essential unknown, a gap in our knowledge that is unlikely to be addressed. Recent evidence has also raised the possibility of species differences in the role of KNDy peptides in the control of episodic GnRH secretion between rodents and ruminants.[417,418] It will thus be important to determine which model applies in other species, including primates. One can hope that application of technical developments in genomics, proteomics, and neurobiology to a variety of species in the future can address the current deficit in comparative studies of reproductive neuroendocrine systems.

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CHAPTER

34

Seasonal Regulation of Reproduction in Mammals

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INTRODUCTION

This chapter deals with the mechanisms by which breeding in mammals is synchronized to environmental seasonality. Seasonality is a fundamental feature of the earthly environment, due to basic geophysical considerations: the Earth's orbit around the Sun, and the 23° tilt in the Earth's axis of rotation relative to its plane of solar orbit (Figure 34.1(A)). These considerations lead to an annual cycle of changing solar light exposure experienced over the entire globe, with an amplitude that varies dramatically based upon latitude: at the poles continuous illumination is replaced by continuous darkness at 6 month intervals, while in the equatorial zones the day length and the zenith of the midday sun vary imperceptibly over the course of the year (Figure 34.1(B)). As a consequence of these differences in the annual cycle of illumination (and hence solar energy supply), the amplitude of annual rhythms of environmental seasonality increases dramatically as one travels away from the equator. Seasonality is also seen within the equatorial zones, related to rainfall patterns, but these occur without the predictable periodicity seen at higher latitudes.

Since the raising of young is the most energetically demanding phase of the life history cycle, and is crucial for evolutionary fitness, mammals have evolved mechanisms to ensure that breeding is timed so that parturition occurs in windows of environmental favorability, typically in the spring and summer months. For short gestation species (for example, rabbits or hamsters), this leads to the breeding season being confined to the spring or summer (so-called "long day breeders"), while longer gestation lengths seen in ungulates are often associated with breeding in the preceding autumn (so-called "short day breeders") (Figure 34.2). Understanding the mechanisms determining the timing of the breeding season was a key motivating factor behind the seminal work of Francis Marshall on reproductive physiology more than a century ago.[3] Regardless of when breeding takes place, mammals rely on a photoperiodic readout system built around pineal production of the indole amine hormone, melatonin. Although a link between the pineal and reproduction in humans was suggested as early as the late nineteenth century from clinical studies linking pineal tumors to precocious puberty,[4] the isolation of melatonin as the principal bioactive output of the pineal gland was not achieved until more than half a century later.[5] Moreover, while melatonin had an unambiguous effect on skin color in amphibians (giving it its name), its role as a calendar signal in mammals was not appreciated until the 1980s, with the synthesis of understanding from experiments in long- and short-day breeding seasonal mammals (revealing clear effects of melatonin on breeding not evident in earlier work in nonphotoperiodic laboratory strains of rat). Progress in the last three decades has been impressive: the pharmacological basis of melatonin receptor signal transduction was established in the 1990s, and the importance of sites of melatonin action in the hypothalamus or in the neighboring pars tuberalis (PT) of the anterior pituitary for seasonal endocrine function also became clear. In the last decade, thyroid hormone metabolism within the mediobasal hypothalamus has emerged as the key gatekeeper for seasonal reproductive activation in mammals as well as in birds. In mammals, melatonin engages with hypothalamic thyroid metabolism via a pathway involving a novel paracrine function of thyrotropin

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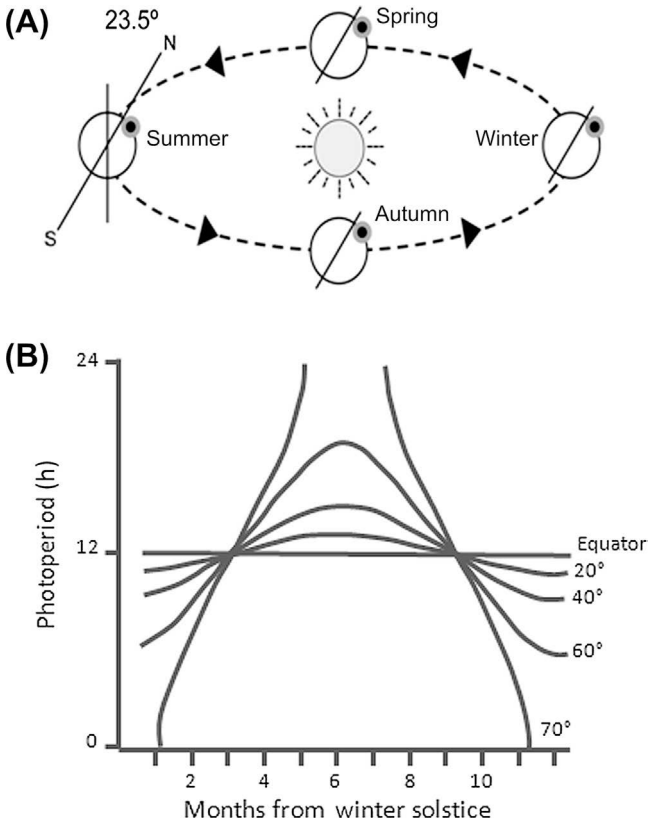

FIGURE 34.1 Geophysical basis of seasonality. (A) The Earth's axis of rotation is tilted at 23° relative to the perpendicular of the plane of rotation around the Sun. The filled point represents a reference location at a high, northern latitude. (B) This gives marked seasonal clines in the amplitude of annual day length variation from an almost constant 12-h day in the equatorial regions to 24 h in the polar regions.

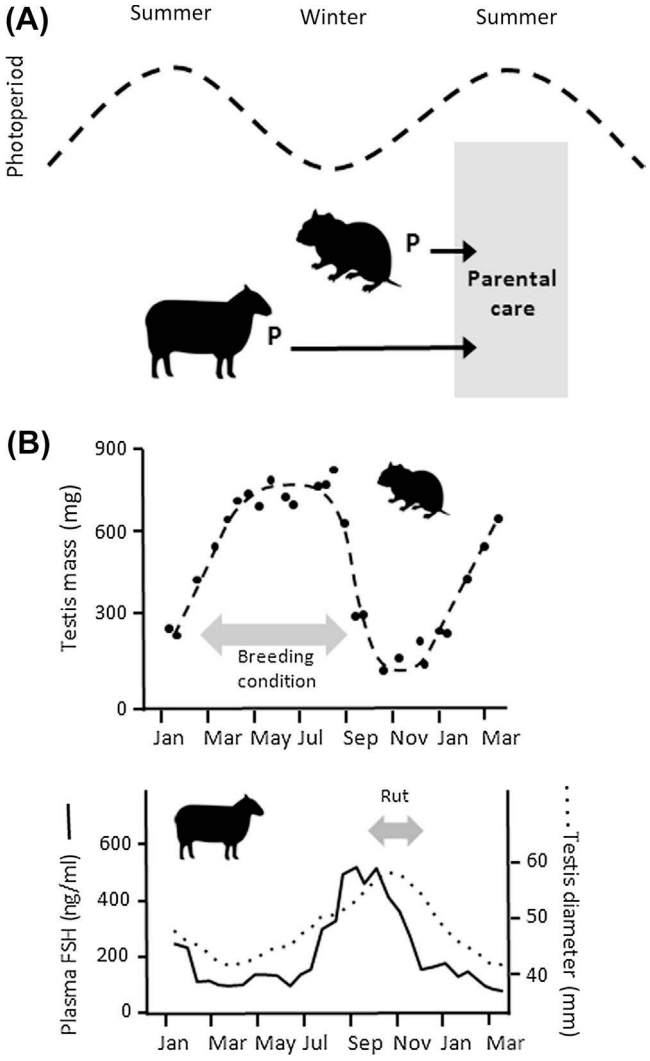

FIGURE 34.2 Timing of the breeding season relative to gestation length. (A) Mammals generally time parturition to coincide with relatively benign spring/summer conditions. This dictates the timing of the breeding season, and hence small rodents with short gestations initiate pregnancy (P) during the summer months, while long gestation ungulates like sheep and deer breed in the preceding autumn. (B) This is reflected in seasonal patterns of testicular growth and regression in male Djungarian hamsters (top panel) and Soay rams (bottom panel) exposed to natural illumination. *Source: Adapted from Refs 1,2.*

(TSH) produced by melatonin-sensitive cells in the PT. Remarkably, this regulatory pathway also operates in birds, although in this vertebrate group photoperiodic input does not rely on melatonin. Current attention now focuses on mechanisms downstream of thyroid metabolism, in particular the role specialized glial cells found around the third ventricle, known as tanycytes, and of hypothalamic neurons expressing kisspeptin (Kp) or argenine-phenylalanine-amide related peptide (RFRP) in mediating changes in gonadotropin-releasing hormone (GnRH) secretion. In this chapter, we discuss the following: the ecological context for seasonal breeding strategies, current knowledge of the mammalian photoperiodic response pathway, and lastly future prospects in this fascinating subject.

ECOLOGICAL CONTEXT FOR SEASONAL BREEDING: ULTIMATE FACTORS AND PROXIMATE CUES

As the driver of seasonal energy availability, the Earth's orbit of the Sun can be seen as the ultimate factor driving the evolution of seasonal breeding strategies. Additionally, this orbit leads to an annual cycle of varying day length (photoperiod), which has become the proximate cue used by seasonal mammals to synchronize breeding to the annual environmental cycle. The exploitation of photoperiod as a cue reflects its invariant cyclical nature (i.e., the hours of daylight on any given date is the same from year to year), which because of its geophysical basis, is reliably predictive. In contrast, other proximate cues such as food availability, temperature, or availability of mates in breeding condition are noisy signals of poor predictive value for forthcoming conditions.

The extent to which breeding is organized to a specific phase of the calendar year may vary in a graded fashion across the latitudinal range occupied by a given species;

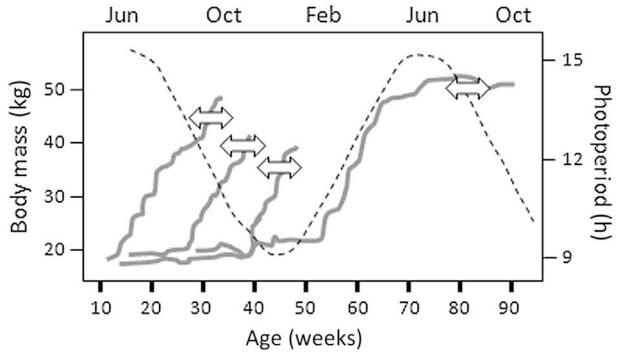

FIGURE 34.3 Nutrition x photoperiod interactions controlling puberty in Suffolk lambs. Shown are the growth trajectories (gray lines) for groups of lambs held on four different feeding regimes. The horizontal double arrows show the timing of puberty in each group. The dashed curve shows the annual photoperiod cycle. Lambs fed from weaning on an ad libitum plane of nutrition grow to about 40 kg by early autumn when they enter puberty. Delaying growth by undernutrition until the autumn or early winter leads to a delayed puberty in the first winter of life, at a reduced body weight compared to ad libitum controls. If, however, growth is delayed by undernutrition until the following spring, then puberty is not attained until the second autumn of life, when body weight is significantly higher than in control lambs. Hence nutrition and photoperiod jointly determine the onset of reproductive maturation in lambs. *Source: Redrawn from Ref. 7.*

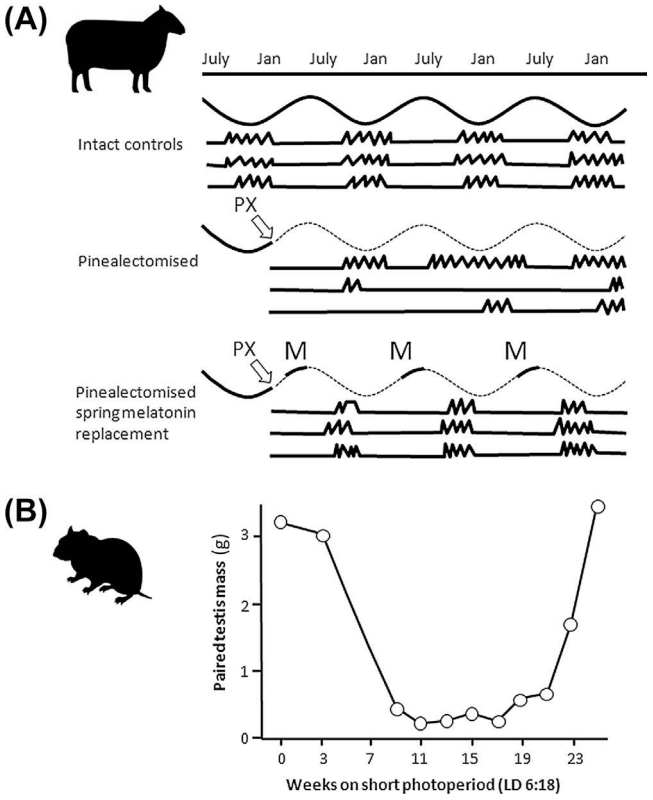

FIGURE 34.4 Innate long-term regulation of reproductive activity in seasonal breeders and the synchronizing effect of melatonin. (A) Ewes exposed to natural cycles of changing day length enter breeding condition synchronously in the autumn-to-winter months. Pinealectomy (PX) removes the endogenous melatonin (M) signal and leads to asynchronous episodes of reproductive activation (squiggles) and quiescence (straight lines). Synchrony can be restored by a subcutaneous infusion of melatonin in a temporal pattern to imitate the endogenous sequence of nocturnal pineal melatonin signals spanning just a quarter of the overall annual cycle. A sequence of infusions imitating the spring-to-summer photoperiod sequence is particularly effective as a circannual synchronizing signal. *(Source: Based on data in Ref. 10.)* (B) Male adult Syrian hamsters exposed to a short photoperiod of 6 h light/18 h darkness for 25 weeks initially undergo testicular regression, reflecting reproductive quiescence. Afterwards, despite continued short day exposure, they display a spontaneous recrudescence of testicular growth and return of reproductive activity. *Source: Redrawn from Ref. 11.*

populations living at higher latitudes showing strong cycles of seasonality consistently synchronized to a specific phase of the calendar year, while lower latitudes favor continuous breeding, with an opportunist element related to prevailing food availability.[6] This pattern suggests that, in the wild, the "decision" to breed is conditional on photoperiod integrated with other proximate cues reflecting energy availability. A nice demonstration of the interaction between photoperiod and nutrition in a laboratory setting can be found in studies by Foster and colleagues of the timing of puberty in sheep[7] (Figure 34.3): for spring-born lambs, puberty onset occurs in the first autumn of life, but this may be delayed by food restriction earlier in life. If release from food restriction occurs soon enough to permit sufficient growth in the first growing season, lambs attain puberty in the first year with a slight delay, and a slightly reduced size, compared to control animals. If, however, feed restriction is extended, delaying growth within the first growing season so that a threshold body size is not achieved in the first autumn of life, puberty is delayed for a full year until the next seasonally permissive window for breeding.

The concept of nutrition × photoperiod interaction is underlined by reproductive behavior in microtine rodents (voles), wherein breeding is highly sensitive to the presence of new growing grasses in the diet.[8] This effect has been proposed to depend on the presence of plant metabolites that act as reproductive stimuli (e.g., Ref. 9), but the precise mechanisms of action of such molecules, and how they interact with the photoperiodic response mechanisms discussed later in this chapter, remains unclear.

Relationship between Photoperiodic Cues and Innate Long-term (Circannual) Timing

A remarkable feature of mammalian seasonal biology is that long-term transitions in seasonal state may proceed independently of changing environmental stimuli. In their most striking manifestation, this property is expressed as so-called "circannual rhythms" of reproduction or energy metabolism in animals held under artificially constant conditions for periods of years (Figure 34.4(A)). The term *circannual* derives from the Latin c., approximately, and *annus*, year, and refers to innate biological rhythmicity with a period length of approximately 1 year. This definition emphasizes the analogy with the more widely studied

"circadian" biology, which concerns innate rhythmicity with a period length of approximately 1 day. Circadian rhythmicity emerges from cellular oscillations in gene transcription and from the existence of pacemaker tissues capable of coordinating circadian rhythms in physiology throughout the organism.[12,13] Efforts to isolate analogous structures responsible for circannual rhythm generation have been less conclusive but suggest that sites within the hypothalamo-pituitary axis may be important.[14] The fundamental origins of circannual rhythm generation have been suggested to derive from cyclical histogenic mechanisms,[15] but the definitive evidence for this is still lacking.

The expression of circannual rhythms necessarily is confined to species whose life history normally encompasses survival through multiple breeding years. In short-lived rodent species, in which adult survival is unlikely to extend into a second winter of life, spontaneous long-term cycling between breeding and nonbreeding condition is not observed. Nonetheless, in these species, too, innate long-term timing mechanisms governing reproductive control are evident and appear to be important for the spontaneous reversion to a spring phenotype (typically including reproductive reactivation, since this group of animals is typically of short gestation length) following prolonged exposure to winter day lengths[11,16,17](Figure 34.4(B)). This phenomenon, which has widely been referred to as *photorefractoriness*, does not involve a loss of capacity to sense day length per se but rather a decoupling between proximal day length–sensing mechanisms and downstream seasonal biology.[18–20] More recent work also demonstrates that the timing and pattern of day-length increase shapes the response to declining day length in Djungarian hamsters (*Phodopus sungorus*).[21] Indeed it has been suggested that the mechanisms underlying photorefractoriness in short-lived species and circannual rhythm expression in long-lived species are probably very similar.[15]

In an ecological context, the spontaneous initiation of long-term changes in reproductive physiology exemplified in Figure 34.4 is generally believed to serve an adaptive function by anticipating forthcoming environmental change. Given that the growth of gonads and of secondary sexual characteristics essential for breeding success may take months to achieve, this interpretation seems justified. Moreover, periods of breeding activity or quiescence may traverse phases of the annual cycle (around the solstices) when rates of seasonal change do not provide a strong seasonal *zeitgeber* (synchronizing cue, from the German *zeit* = time and *geber* = giver), and hence favor internal long-term timers to drive predictive seasonal change. In hibernating species, winter survival may rely on behaviorally driven isolation from external seasonal time cues while sheltering in the hibernaculum, and this would further militate in favor of evolving internal long-term timing mechanisms. While this adaptive reasoning is entirely plausible, formal tests of the evolutionary fitness advantage of innate long-term timing have not been attempted.

Lastly, it should be noted that in equatorial species, it appears that innate long-term timing of seasonal breeding rhythms occurs independently of photoperiodic input, and is instead reliant on rainfall or conspecific social cues for its synchronization.[15]

Photoperiodic History Dependence and Maternal Photoperiodic Programming

Associated with the concept of circannual rhythmicity is the concept of photoperiodic history–dependence (i.e., that the response to exposure to a given day length depends on the prior sequence of day length experienced by the animal). A clear rationale for predicting that photoperiodic sensing will be history dependent is the response required to equinoctial day lengths experienced in the spring and autumn—the former being predictive of summer, the latter of winter. Evidence for history-dependent responses at the level of overt reproductive activation comes from studies in rodents and in ungulates. For example, in the Djungarian hamster, exposure to a 14-h day length will either stimulate reproductive activation or regression depending upon whether prior exposure was to 16-h or 10-h.[22]

The importance of history-dependent photoperiodic programming applies also to postnatal reproductive development in juvenile animals. Photoperiodic information is relayed transplacentally, giving a photoperiodic history reference to determine appropriate photoperiodic responses in the juvenile period[23,24](Figure 34.5). For rodent species in which growth and development proceed rapidly in the first growing season, allowing progeny to breed in the same year in which they are born, the ecological significance of maternal photoperiodic programming is clear: a program of accelerated reproductive development is appropriate for spring-born offspring, while for late summer/autumn-born progeny delaying breeding to the following spring is the only viable strategy.[24] Indeed, laboratory-based studies of photoperiodic programming mechanisms were initially stimulated by field data demonstrating a strong month of birth effect on rate of sexual maturation in montane voles (*Microtus montanus*).[8] The mechanisms behind this transplacental relay of photoperiodic information probably rely on the pineal hormone, melatonin, since pinealectomy or melatonin injections have pronounced effects on photoperiodic programming.[23] Maternal photoperiodic programming effects have also been observed in the sheep, with prolactin secretion in lambs being highly sensitive to photoperiodic experience in utero[7] (Figure 34.5(B)). These programming effects emphasize the profound importance of melatonin for seasonal photoperiodic synchronization in mammals, discussed in detail in the following section.

The mechanisms underlying photoperiodic history dependence may be similar to those involved in circannual rhythm expression, and as such may lie downstream of the proximal day length sensing pathway. Nonetheless

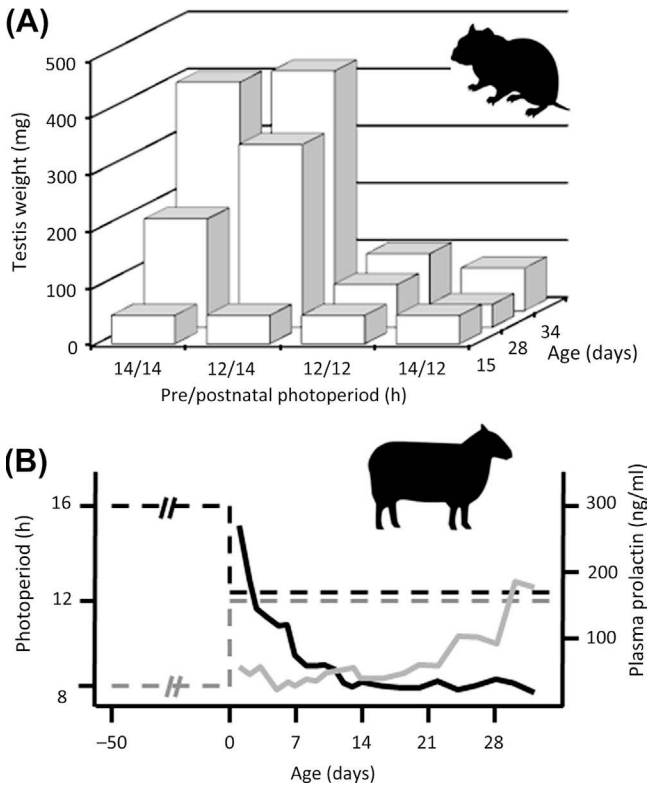

FIGURE 34.5 Maternal photoperiodic programming of reproductive development and endocrine function. (A) In Djungarian hamsters, maternal exposure to different combinations of 12-h and 14-h photoperiods during gestation and postnatally leads to widely different rates of gonadal growth (testis mass at 28 days). Additionally, exposure to a 14-h photoperiod during postnatal life promotes more rapid gonadal development, if the prenatal photoperiod was 12 h rather than 14 h. Hence maternal photoperiodic history programs photosensitivity in the neonate. *(Source: Graph drawn based on data in Ref. 25.)* (B) In Suffolk lambs, prolactin secretion shows a similar history dependence. Exposure to an equinoctial photoperiod for the first 28 days of postnatal life suppresses prolactin secretion in lambs born to mothers held on a 16-h photoperiod, but stimulates it in lambs born to mothers held on an 8-h photoperiod. *Source: Redrawn from Ref. 7.*

perinatal light exposure in rodents has been reported to alter day length responses in the suprachiasmatic nuclei (SCN) and in the pineal gland, both of which constitute important upstream elements in the photoperiodic response pathway, as we will discuss in detail following. Hence history-dependent programming of seasonal light responses probably acts at multiple levels.

ROLE OF THE PINEAL HORMONE MELATONIN IN MAMMALIAN SEASONAL BREEDING

At the end of the nineteenth century, Heubner presented the case of a boy with pineal tumor associated with precocious puberty,[4] suggesting a link between the pineal and reproduction. In 1917 McCord and Allen[26] described the skin-lightening effect of feeding tadpoles a pineal extract. This effect formed the bioassay for the eventual isolation of *N*-acetyl-5-methoxytryptamine some four decades later, and led to the name *melatonin* being coined for this indole amine.[5] Although effects of pineal extracts on reproduction in rats had been described prior to the isolation of melatonin (e.g., Ref. [27]), the pivotal role of melatonin in the regulation of reproductive activity became clear only when studies were performed in seasonally breeding rodents.[28–30] In the male Syrian hamster, pinealectomy or pineal denervation prevented short day–induced gonadal regression, and this effect was reversed by melatonin administration,[29,31,32] suggesting an "antigonadal" role for melatonin. Subsequent experiments in short day breeding ungulates further supported a link between the pineal gland and photoperiodic synchronization, but refuted the antigonadal concept.[33–35]

Although melatonin is synthesized in sites including the retina, Harderian gland, and gut,[36–39] levels of melatonin in the circulation[40] and in the cerebrospinal fluid (CSF)[41] are dependent entirely upon synthesis in the pineal gland. The mammalian pineal gland develops as an evagination of the diencephalic roof. In some species like sheep and humans, the gland forms a solid mass adjacent to the third ventricle, and part of the synthesized melatonin is therefore released into the CSF, giving nighttime CSF concentrations some two orders of magnitude higher than in the peripheral circulation.[41] By contrast, in rodents, the gland consists of a minor part closely related to the third ventricle, whereas the major part has migrated dorsally between the cerebral hemispheres and the cerebellum. As we shall see later, melatonin synthesis in the pineal is tightly temporally controlled, allowing melatonin to uniquely serve as a hormonal representation of time.

Control of Pineal Gland Activity

Pineal melatonin synthesis displays a marked daily rhythm that is restricted to the dark phase of the day.[42,43] The rhythmic synthesis of melatonin depends upon three interdependent factors: the endogenous circadian oscillator located in the SCN, the light/dark cycle that synchronizes the circadian oscillator, and the acute inhibition of melatonin synthesis by light.[44–47] The complex pathway controlling the pineal gland, termed the *retino-hypothalamo-pineal pathway*, ends with dense sympathetic innervation of the pineal parenchyma[48–50] (Figure 34.6). Photic information is conveyed to the SCN principally via the retino-hypothalamic tract, which relies on signals from conventional rod and cone photoreceptors as well as from a specialized subset of retinal ganglion cells, which are intrinsically light sensitive. These ganglion cells express the photopigment melanopsin, which is maximally sensitive to light in the blue region of the spectrum (@ 485 nm), and project to the SCN where they release

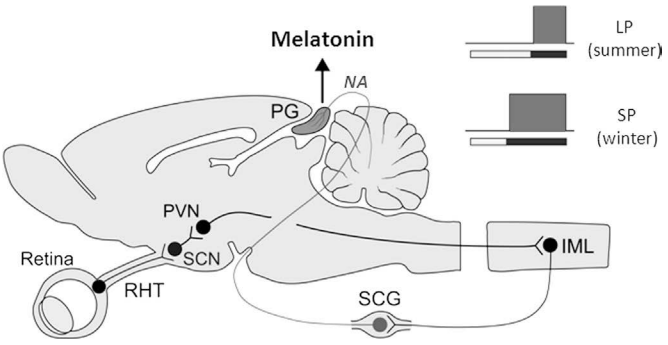

FIGURE 34.6 The photoneuroendocrine system drives the rhythmic release of melatonin. In mammals, light/dark information is perceived by photoreceptors and retinal ganglion cells that project, via the retinohypothalamic tract (RHT), to the suprachiasmatic nuclei of the hypothalamus (SCN), seat of the master circadian clock. In turn, the SCN controls the activity of the pineal gland (PG) via a polysynaptic pathway running via the paraventricular nucleus of the hypothalamus (PVN), the intermediolateral cells of the spinal cord (IML), and the superior cervical ganglia, whence noradrenergic neurons project massively to the pineal gland. The pathway is shown on a schematic parasagittal section of rodent brain. The activity of the SCN restricts noradrenaline (NA) release and, consequently, melatonin synthesis and secretion to the night time. The activity of the SCN is regulated by day length (photoperiod), and as a consequence the duration of the nocturnal melatonin message changes seasonally: during summer, long photoperiods (LP) are associated with short melatonin peaks, whereas during winter, short photoperiods (SP) are associated with longer melatonin peaks. *Source: modified from Ref. 51.*

the neurotransmitters glutamate and pituitary adenylate cyclase activating peptide (PACAP), which mediate light entrainment effects on the SCN circadian pacemaker.[52–54] The molecular clockwork generating and synchronizing the circadian activity within each SCN cell has been well established and relies on a combination of cell autonomous circadian oscillators based on transcriptional negative and positive feedback loops involving a set of clock genes (particularly *Clock*, *Bmal1*, *Per1-3*, *Cry1-2*, *Rev-erbα*, *Rorβ*), as well as extensive coupling between SCN neurons through VIP/PACAP signaling.[55–57] Importantly in the context of seasonality, daily rhythms of clock gene expression in the SCN (Figure 34.7) respond to annual changes in photoperiod,[58–62] with diurnally expressed genes showing extended periods of elevated expression in animals exposed to long photoperiod. This is associated with altered sensitivity to light input, as observed through light-induced c-fos expression, as well as modulation of rhythmic SCN outputs, including vasopressin synthesis[58,63] and electrical activity.[64] In turn, these effects are presumed to modify rhythmical behavior of SCN targets, which include the pineal gland. The hypothalamic paraventricular nuclei act as an essential relay between the SCN and pineal melatonin synthesis, exerting a sustained tonic stimulation of pineal activity during the night, which is inhibited during the day by the SCN-driven release of GABA.[65,66] Hypothalamic timing information is forwarded to the intermediolateral cells of the upper three segments of the spinal cord and the superior

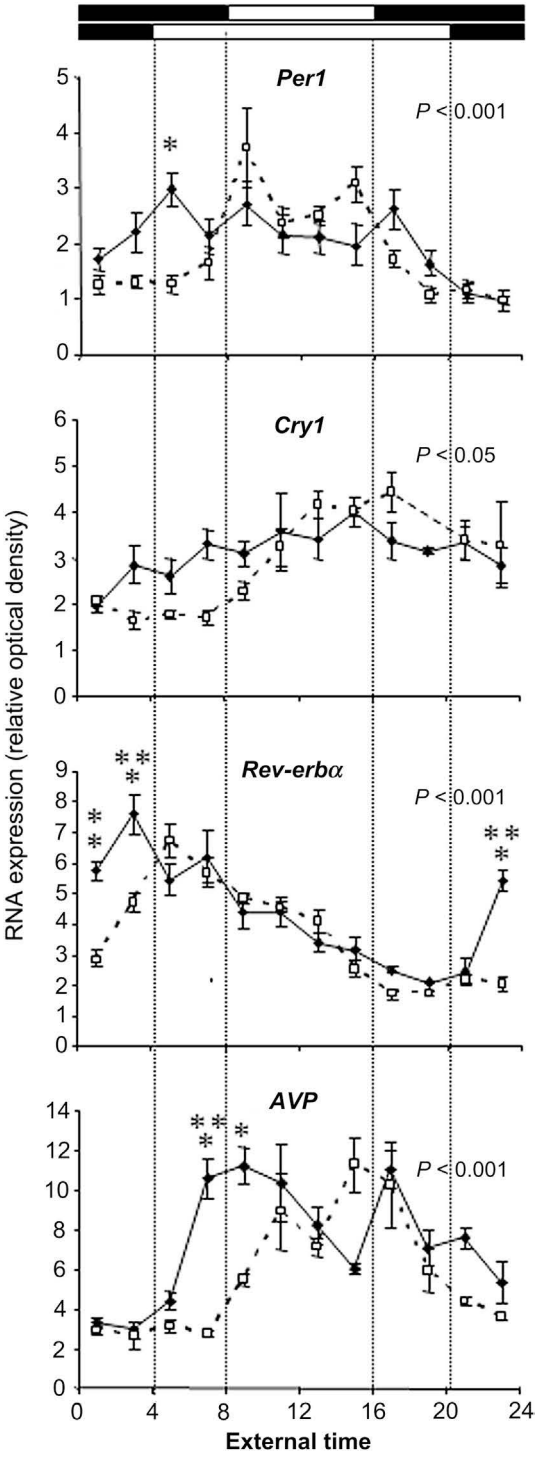

FIGURE 34.7 The SCN encodes photoperiodic variation in day length. Photoperiod modulates rhythmic expression of multiple genes in the Djungarian hamster SCN. The graphs show the 24-h expression profiles of clock and clock-controlled gene mRNA in the hamster SCN. Animals were housed under long photoperiod (filled diamonds, solid line) or short photoperiod (open squares, dotted line) for 8 weeks. Each value is mean ± SEM of four animals. Solid and open bars represent the dark and light periods, respectively. Clock genes: *Per1* = period 1, *Cry1* = cryptochrome 1, *Rev-erbα* = nuclear receptor subfamily 1, group D, member1; clock-controlled gene: *AVP* = argenine vasopressin. *P*-values represent interaction between Photoperiod and External Time (12.00 = mid light phase) for each gene. *Source: Modified from Ref. 58.*

cervical ganglia and finally reaches the pineal gland as a marked nocturnal release of norepinephrine (NE).[67–69] The pineal gland receives other fibers from various origins and containing various neurotransmitters, but NE is by far the most potent and critical neurotransmitter to generate the rhythms in melatonin synthesis.[70]

Melatonin Synthesis Pathway

The effect of NE on the pineal biochemistry has mainly been studied in the rat, but it should be kept in mind that species differences exist in the cellular pathways activated by NE at night. Activation of the pineal β1- and α1-adrenergic receptors induces a large increase in intracellular levels cAMP and Ca^{2+} and a downstream activation of cAMP dependant protein kinase A (PKA), a pivotal cellular event leading to melatonin synthesis at night.[70–73] The multistep enzymatic pathway through which NE controls melatonin synthesis is summarized in Figure 34.8. Melatonin synthesis is initiated from circulating tryptophan taken up from the blood stream and metabolized into 5 hydroxytryptamine (5HT) by the consecutive action of tryptophan hydroxylase and aromatic amino acid decarboxylase, whose activities display negligible daily and seasonal changes. By contrast, the subsequent enzyme, which converts 5HT into *N*-acetylserotonin, arylalkylamine-*N*-acetyltransferase (AANAT), exhibits a dramatic nighttime increase in activity in all species investigated.[43,68,70,74–78] The final step of melatonin synthesis is catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT), the activity of which displays negligible daily variations.[42,79,80]

There was an interval of about 25 years between the discovery of the pivotal role of AANAT in driving the nocturnal increase in melatonin synthesis and the delineation of the molecular pathways underlying this activation.[81–83] Analysis of *Aanat* transcriptional regulation shows remarkable species differences. In rodent species, the *Aanat* gene is not expressed during the daytime; but at the beginning of the night, following NE release and PKA activation, there is a large increase in *Aanat* mRNA levels followed by a synthesis in AANAT protein, the latter being activated by phosphorylation and interaction with the 14-3-3 protein. Contrastingly, in ungulates, primates, and humans, the *Aanat* gene is constitutively expressed throughout day and night, but during the day the AANAT protein is immediately degraded by proteolysis, whereas at night phosphorylation of AANAT leads to its activation and stabilization by interaction with 14-3-3 protein[70,84–86] (Figure 34.9). The two major features of AANAT are its marked activation upon nighttime release of NE and its very short half-life (3–5 min), leading to a rapid decrease upon termination of NE stimulation at the end of the night or following light exposure at night.[47,90,91] As a consequence, AANAT behaves as an ON/OFF signal and should be viewed as the *melatonin rhythm generating enzyme* and the regulator of the nocturnal melatonin

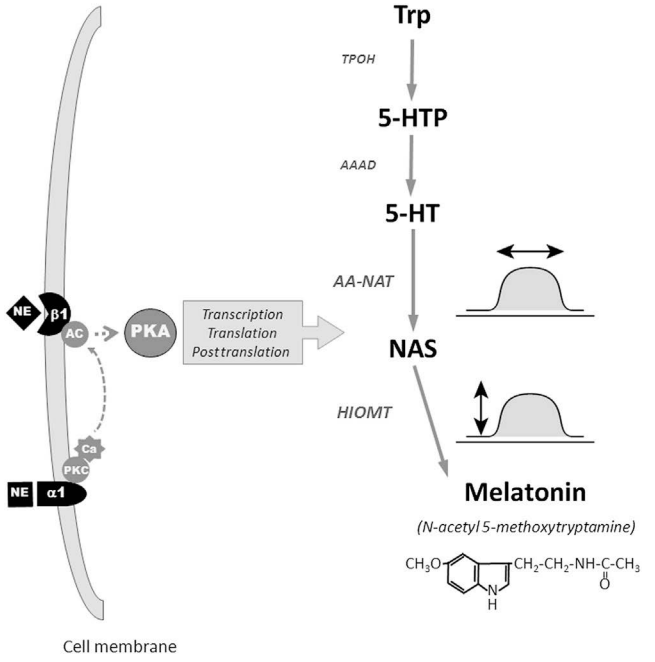

FIGURE 34.8 Biochemical pathway of melatonin synthesis. Melatonin is synthesized from tryptophan, which is metabolized into serotonin (5-HT) by tryptophan hydroxylase (TPOH) and aromatic amino acid decarboxylase (AAAD); serotonin is next acetylated into *N*-acetyl serotonin (NAS), then methylated into melatonin by the consecutive action of the pineal-specific arylalkylamine *N*-acetyl transferase (AA-NAT) and hydroxyindole-*O*-methyltransferase (HIOMT). Norepinephrine (NE) released at night binds to β1- and α1-adrenergic receptors leading to a strong activation of a cAMP-dependent protein kinase A (PKA). PKA acts through multiple biochemical pathways to promote melatonin synthesis. Notably, AANAT enzyme activity is strongly activated at night onset and inhibited at night offset, determining the duration of the nocturnal synthesis of melatonin. Contrastingly, HIOMT activity shows only a weak nocturnal induction but displays photoperiodic changes in activity with higher values in short photoperiod. This may modulate the amplitude of nocturnal melatonin synthesis.

peak duration.[92,93] Although the activity of HIOMT does not show daily variations,[42,79,80] there is a clear photoperiodic/seasonal variation in the enzyme activity being higher in short as compared to long photoperiod.[89,94] This long-term regulation of HIOMT activity may result from extended *Hiomt* (also known as *Asmt*) transcription during long nights combined with high protein stability. Strikingly, in seasonal species like the Djungarian hamster, higher HIOMT activity in short photoperiod is clearly associated with heightened nighttime production of melatonin, leading to the proposal that, during the night, HIOMT is the *melatonin rate limiting enzyme*.[89] Hence, the combination the two melatonin synthesizing enzymes with differing biochemical characteristics, the reactive but unstable AANAT together with the stable HIOMT, shapes the daily and seasonal rhythm in melatonin synthesis (Figure 34.8).

Melatonin is not stored in the pineal cells but directly released by diffusion. In all species investigated, the pineal gland is a highly vascularized organ[95] and has

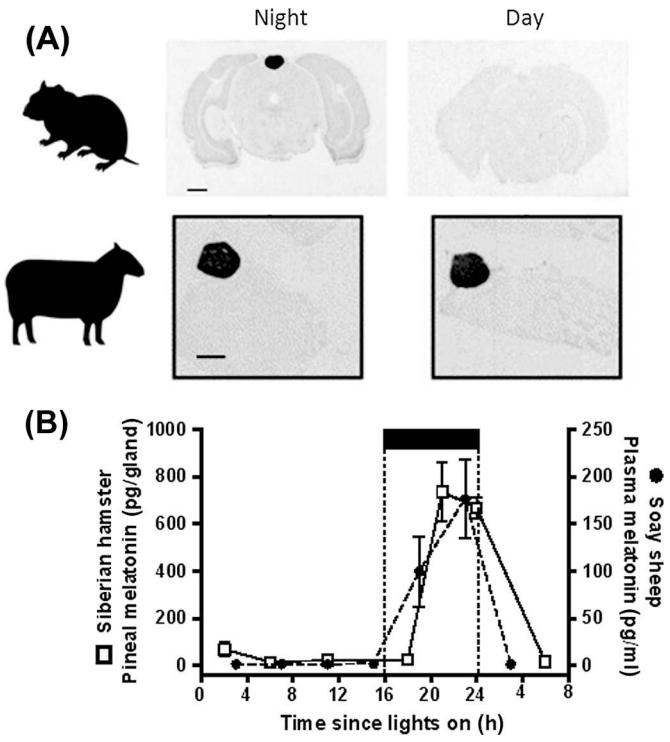

FIGURE 34.9 Regulation of pineal AANAT gene expression in rodents and ungulates. (A) Radioactive in situ hybridizations showing that *Aanat* mRNA levels are markedly increased at night in the rat pineal gland (top panel), *(Source: Adapted from Ref. 87.)* whereas they are constitutively elevated in the sheep pineal gland (bottom panel) *(Source: Adapted from Ref. 88.)*; scale bars are 1 mm and 5 mm, respectively. (B) The differential regulation in *Aanat* gene expression results in an earlier onset of melatonin synthesis and secretion at night onset in ungulates *(Source: Data here from the Soay sheep from Ref. 19.)*, where AANAT activity is regulated posttranslationally, as compared to rodents. *(Source: Data here in the Djungarian hamster, adapted from Ref. 89.)*

a blood flow estimated to be 4 ml/min/g.[96] Circulating melatonin is rapidly degraded by hepatic hydroxylation into 6 hydroxymelatonin and then excreted in the urine mainly as 6 sulfatoxymelatonin, with a half-life of approximately 20 min.[97,98] Any modification in the synthesis pathway is thus immediately translated into similar modification in circulating melatonin. Because the duration of the nocturnal synthesis of melatonin is positively related to the length of the night, the changes in circulating melatonin constitute a strong endocrine representation of the time of the day and time of the year. Notably, the daily profile of circulating melatonin may differ greatly among species, and even among individuals of the same species. For example, in the rat, the plasma melatonin concentration increases from about 10 pg/ml at daytime up to 50–500 pg/ml at nighttime,[70,99–101] whereas in the sheep, the nighttime peak ranges from 100 to 1000 pg/ml.[102] Notably, however, under stable photoperiodic conditions the melatonin pattern is highly reproducible in the same individual.[103,104]

Timed Infusion Experiments Defining Melatonin's Mode of Action

Based on the elucidation of the pattern of synthesis and secretion of melatonin by the pineal gland, several groups undertook experiments aimed at defining how photoperiodic information was encoded in the melatonin signal. The key approach has been to combine pinealectomy (PX) to ablate the endogenous circulating melatonin signal with timed subcutaneous infusion of melatonin to mimic melatonin secretion under different photoperiodic conditions. This has been dubbed the "timed-infusion paradigm."[105] Three basic hypotheses for information coding in the melatonin signal have been considered: (1) that the total amount of melatonin secretion dictated the response (amplitude hypothesis); (2) that the continuous duration of melatonin secretion dictated the response (durational hypothesis), and (3) that the phase relationship between periods of melatonin secretion and an internal circadian clock dictated the response (melatonin sensitivity rhythm hypothesis). Of these, the overwhelming evidence points toward the duration of the interval between the evening onset and dawn offset of melatonin secretion as the key variable through which photoperiod is encoded (Figure 34.10). Maintained presence of melatonin between these two transitions, and absence of melatonin during daylight hours, appear also to be key features.[105–110] There is no absolute requirement for the period of elevated melatonin infusion to synchronize with nighttime in PX hamsters, indicating no requirement for melatonin readout by melatonin-independent aspects of circadian organization.[111] Nonetheless two studies have reported that short breaks in melatonin secretion during the night may not interfere with melatonin signal interpretation.[112,113] While these studies do not refute the basic requirement for maintained presence of melatonin for longer intervals between dusk and dawn, they do hint at a possible melatonin-based oscillator in the readout mechanism; this idea is echoed in subsequent molecular studies on this issue,[62,114] which are discussed below.

As well as defining the signal characteristics of pineal melatonin secretion necessary for photoperiodic synchronization, the timed infusion paradigm has been important for delineating the relationship between this synchronization process and innate long-term/circannual timing. In the sheep, long-term seasonal breeding cycles can be effectively synchronized by melatonin input delivered for only 25% of the year, so that the circannual breeding rhythm effectively free runs for 9 months of the year[10] (Figure 34.4). This approach also highlights the idea that specific phases of the year may be particularly important for photoperiodic

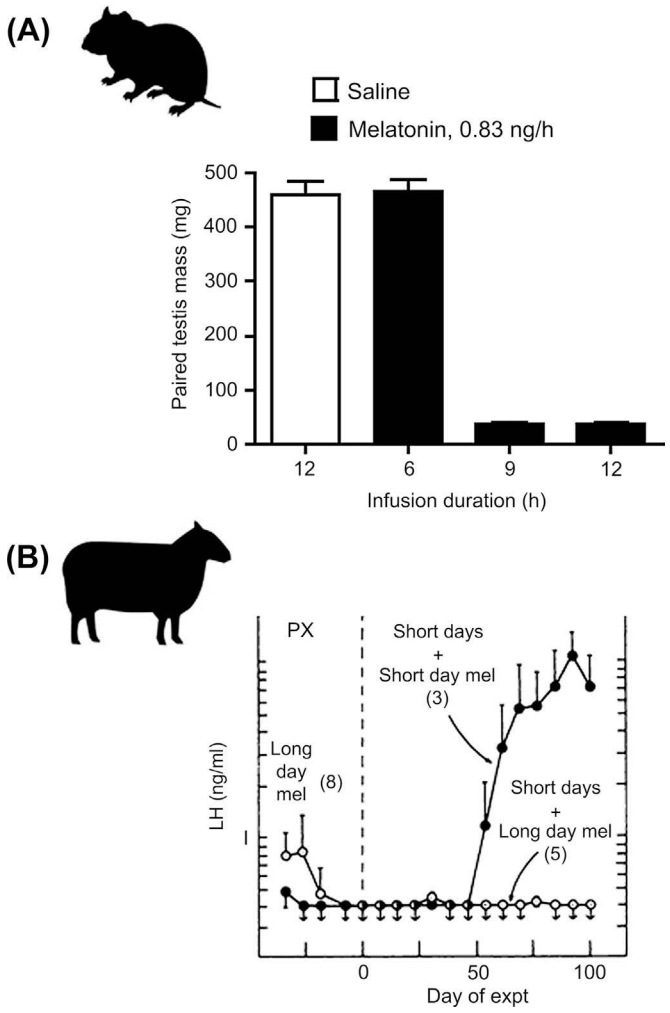

FIGURE 34.10 Timed melatonin infusion experiments demonstrate the importance of melatonin signal duration for photoperiodic responses. (A) Mean paired testis mass measured in long day housed PX male Djungarian hamsters receiving different daily durations of melatonin infusion. *(Source: Adapted from Ref. 106.)* (B) Mean serum LH concentrations in PX and ovariectomized + estradiol-treated ewes treated daily with a short or long melatonin infusion. Prior to day 0, all PX ewes received a long day melatonin infusion; beginning day 0, all ewes were transferred to short days and five ewes continued to receive the long day melatonin pattern (O) while three were switched to a short day melatonin infusion (●). *(Source: Adapted from Ref. 107.)* Note that whereas short day (long duration) melatonin infusions cause gonadal regression in hamsters, they promote reproductive activation in sheep.

synchronization: maintained circannual synchrony is strongest for sheep receiving a melatonin infusion mimicking the increasing day length between the spring equinox and the summer solstice, and weakest for sheep receiving a signal mimicking the declining day length between the autumn equinox and the winter solstice. The concept of photoperiodic/melatonin entrainment of underlying circannual rhythmicity also appears to apply in the European hamster (*Cricetus cricetus*), a long-lived rodent species.[115]

MELATONIN RECEPTORS AND SITES OF ACTION

Consistent with the standard model for pubertal reproductive activation through changes in central sensitivity to gonadal negative feedback signals, as described in Chapter 31, steroid clamping experiments in seasonal sheep demonstrated photoperiodic shifts in the sensitivity of the GnRH pulse generator to gonadal steroids.[116] Gonadal steroid negative feedback effects are strongest with the transition to anestrus and weakest with the onset of the new breeding season. This led to the general inference that seasonal photoperiodic effects of melatonin must be mediated through central, most likely hypothalamic, sites of melatonin action. Early direct evidence for this mode of action came from studies using melatonin-impregnated pellets placed in the hypothalamus of white-footed mice (*Peromyscus leucopus*), resulting in reproductive suppression.[117] The current model outlined below is broadly consistent with a central mode of melatonin action and highlights the role of melatonin sensitive cells not in the hypothalamus, but in the immediately adjacent pituitary stalk, in the cells of the PT.

Binding Studies and Melatonin Receptor Autoradiography

Progress on understanding the sites of melatonin action benefited enormously from the synthesis of an iodinated analog of melatonin, 2-iodomelatonin, which retains biological activity mimicking that of melatonin itself.[118,119] This opened the way for analysis of tissue distribution of melatonin binding sites, their functional characterization, and, ultimately through expression cloning approaches, their molecular characterization.

Using high specific activity radio-iodinated 2-[^{125}I]-iodomelatonin (IMEL) for in vitro autoradiography, it became clear that high affinity melatonin bindings sites were present in the hypothalamus and PT of mammals.[120–123] Considerable species variability in the distribution of brain IMEL binding sites has emerged, with hypothalamic sites including the SCN, dorso- and ventro-medial hypothalamic nuclei, mediobasal hypothalamus, and premamillary arcuate nucleus (ARC) having been recorded in many instances, along with a variety of sites elsewhere in the central nervous system.[121,124,125] Additionally, high levels of melatonin binding sites have been consistently observed in the PT of the pituitary stalk but not in the other portions of the anterior pituitary (i.e., the pars distalis, PD)—a finding seen in all mammals studied to date[122,126–133] with the possible exception of humans[134] (Figure 34.11). In these studies, levels of IMEL binding in the PT consistently exceed those seen in any neighboring brain area.

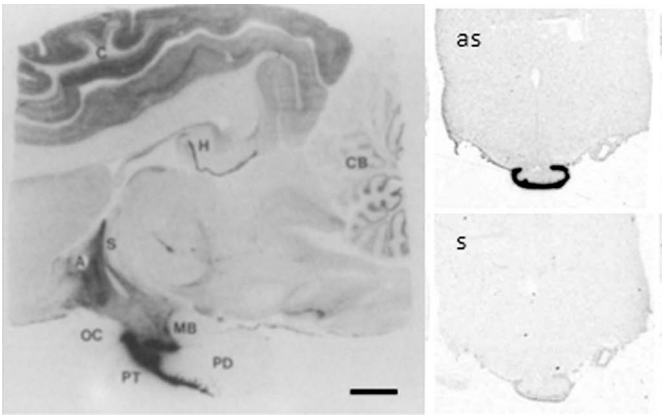

FIGURE 34.11 The pars tuberalis (PT) expresses a high density of melatonin receptors. Staining for melatonin receptor distribution in the sheep brain and pituitary gland. Left panel: autoradiography for high affinity melatonin binding sites in a parasagittal section, using the radioanalog of melatonin 2-[^{125}I]-iodomelatonin (IMEL). Note the intense labeling in the pituitary pars tuberalis (PT), and lower levels of labeling in the hypothalamus (between the optic chiasm (OC) and the mammillary body (MB)), the cortex (C), and the cerebellum (CB). *(Source: Modified from Ref. 135.)* Right panel: In situ hybridization for type 1 melatonin receptor (*Mtnr1a*) RNA expression in coronal sections through the sheep hypothalamus. Note the intense labeling with the antisense (as) probe in the PT region. Scale bar = 5 mm. *Source: Images from Dr Hugues Dardente.*

Pharmacological characterization of the IMEL binding sites in the PT as well as in the hypothalamus indicated that they represented high affinity (Kd in the picomolar range) G protein–coupled receptors (GPCRs).[124] Further characterization of these receptors, principally using cells harvested from ovine PT tissue, revealed distinct pharmacological subtypes and coupling to various signal transduction pathways. Of these, acute inhibitory and chronic sensitizing effects of melatonin on adenylyl cyclase/cAMP signaling have been most extensively characterized,[136–139] while effects on intracellular calcium levels,[136,140] on arachidonic acid,[141] and on phosphoinositide[142] signaling have also been described. Downstream effects on cAMP-dependent protein kinase, and MAPK, as well as on phosphorylation-dependent transcription factors have also been described.[143–145]

Cloning of the G Protein-Coupled Melatonin Receptor Family

The development of IMEL also opened the way for cloning of the melatonin receptor using an expression cloning approach and RNA from *Xenopus laevis* dermal melanophores,[146] and this led to the cloning of mammalian and avian orthologs based on sequence homology.[147,148] Three melatonin receptor subtypes have been characterized and designated (see http://www.iuphar-db.org/) MT1 (previously Mel1a), MT2 (previously Mel1b), and Mel1c. Of these, MT1 and MT2 are present in all vertebrate groups, while Mel1c is found only in nonmammalian vertebrates.[149] Contrastingly, peculiar to mammals is the orphan melatonin-related receptor (GPR50), which does not bind melatonin in in vitro expression assays and remains a puzzle from a functional perspective.[150] Cloning of the genes encoding MT1 and MT2 (designated *Mtnr1a* and *Mtnr1b*, respectively, see http://www.genenames.org/) allowed a further characterization of melatonin receptor localization in the mammalian brain and showed that the previously observed high level of IMEL binding in the PT was attributable to *Mtnr1a* expression (Figure 34.11).

Evidence that G Protein-Coupled Melatonin Receptors Mediate Seasonal Neuroendocrine Synchronization

Several lines point toward the seasonal neuroendocrine actions of melatonin being mediated by GPCRs. Firstly, the affinity and functional sensitivity of MT1 and MT2 receptors are both in the picomolar range and correspond well to the observed day–night variation in plasma or CSF melatonin levels (plasma nighttime maximum @ 100–300 pM; CSF nighttime maximum @ 1 nM in sheep). This contrasts with the various proposed non-GPCR routes of melatonin action that appear reliant on concentrations of melatonin well in excess of the nighttime peak driven by pineal melatonin secretion. These GPCR-independent routes of melatonin action include antioxidant actions,[151] low affinity interactions with a quinone reductase,[152] and the controversial assignment of RORβ as a nuclear melatonin receptor.[153,154]

Secondly data from mice in which the effects of melatonin receptor knockout have been explored also favor a GPCR-mediated route of melatonin action. Laboratory mice do not show overt seasonal photoperiodism, probably because of many generations of inbreeding in animal facilities run on a constant (often 12-h) photoperiod. Nevertheless, they do express melatonin receptors in the hypothalamus and pituitary[155,156] and have proved useful models for understanding initial mechanistic steps in photoperiod sensing. In particular, photoperiod/melatonin effects on pituitary and hypothalamic gene expression seen in seasonal mammals are also seen in mice and appear to depend on presence of the MT1 receptor.[157,158] Knockout of *Mtnr1a* abolishes these transcriptional effects and all IMEL binding in the mouse PT, a result that not only emphasizes the importance of GPCRs for melatonin signaling but also of the MT1 receptor in particular. Intriguingly, in the Djungarian hamster, which shows strong seasonal photoperiodic control of breeding, the MT2 receptor appears not to be expressed, due to in-frame STOP codons in the *Mtnr1b* gene predicted to grossly truncate the translated receptor protein.[159] This

species nonetheless shows high levels of IMEL binding in the PT and appears to use the same downstream pathways to control seasonal phenotype as seen in other mammals. Hence data from both laboratory and natural melatonin receptor knockouts favor the interpretation that the key receptor for seasonal neuroendocrine control is MT1. With the exception of the retina, where both MT1 and MT2 are highly expressed, typically MT1 appears to be the dominantly expressed form based on in situ hybridization or RT-PCR analysis, with MT2 present at significantly lower levels.[160,161] It remains to be seen whether these two receptor isoforms play distinctive functional roles in mammals.

A Link between the Melatonin-Related Receptor/GPR50 and Energy Homeostasis

Recently it was shown by synteny mapping that GPR50 is the mammalian ortholog of MEL1c.[162] Rapid evolutionary divergence from the ancestral MEL1c appears to have occurred with the branching of the mammalian lineage, and in the duck-billed platypus a MEL1c-like MEL1c/GPR50 is found. The underlying causes for this divergence are unclear, but are most simply interpreted as a consequence of a major chromosomal rearrangement, resulting from GPR50 residing near the end of the long arm of the X chromosome in eutherian mammals. X-chromosomal genes are typically associated with sequence instability, and the syntenic neighbors of GPR50 show a similar rapid evolution.[162] Despite this, GPR50 shows highly localized expression in the mammalian brain, consistent with maintained function, and evolutionary sequence analysis suggests that GPR50 has been subject to directional selection in mammals, rather than just neutral drift on an evolutionary pathway to a loss of function.[162]

These reflections get us little closer to establishing a function for GPR50 in mammals or to identifying possible ligands through which it may exert a signaling function. One school of thought, propounded by Jöckers and colleagues,[163] is that GPR50 acts as a dimerization partner for mammalian melatonin receptors, thereby modulating melatonin signal transduction in a ligand-independent manner. A difficulty with this hypothesis is the rather limited anatomical overlap between melatonin receptor and GPR50 expression, the former being concentrated in the PT, the latter showing strongest expression in brain sites in which melatonin receptor expression is weak or absent.[164,165]

In the hypothalamus, GPR50 is expressed in sites that have been linked to energy homeostasis (e.g., dorsomedial hypothalamus and paraventricular hypothalamus),[164] which raises the possibility that this receptor may mediate the well-known seasonal changes in food intake and metabolism.[166] In rodents, GPR50 is also expressed in the ependymal cell layer lining the wall of the third ventricle. In Djungarian hamsters, exposure to short photoperiods leads to a loss of body fat, reproductive switch off, and suppressed GPR50 expression in the mediobasal hypothalamus.[167] Knockout of GPR50 in mice produces a lean phenotype, and entry into torpor in response to food restriction.[168,169] These findings appear to link GPR50 to metabolic regulation, and this is likely to remain the focus in the search for ligands for this orphan receptor.

IDENTIFYING FUNCTIONALLY IMPORTANT SITES OF MELATONIN ACTION

The identification of hypothalamic and pituitary sites of high affinity IMEL binding has informed attempts to identify key sites of melatonin action through site-specific lesioning, microinjection, or microimplant studies.

Pars Tuberalis as a Site of Melatonin Action for Seasonal Control of Prolactin Secretion

The first direct evidence linking the PT to seasonal neuroendocrine control came from studies using the hypothalamo-pituitary disconnection (HPD) technique in sheep, pioneered by Iain Clarke and colleagues.[170] This involves surgically ablating neurosecretory terminals in the median eminence of the hypothalamus, effectively blocking the descending flow of information from hypothalamic hypophysiotropic hormone secreting cells into the anterior pituitary, while leaving the pituitary (including the PT) intact. This produces animals in which normal anterior pituitary hormone regulation is drastically curtailed, with control of the thyroid, somatotropic, gonadal, and glucocorticoid axes largely absent. Remarkably, Lincoln and Clarke[171] demonstrated that seasonal photoperiodic regulation of prolactin secretion persists in HPD rams in a form indistinguishable from that seen in intact animals (Figure 34.12). Importantly, in line with the concept that acute regulation of prolactin does depend on descending hypothalamic signals (for example, TRH or dopamine), the HPD lesion completely abolishes the prolactin response to stress (namely a barking dog). Hence photoperiodically synchronized long-term regulation of prolactin, but not acute, stress-dependent regulation of prolactin persists in HPD rams.

There is no evidence that melatonin receptors are present in anterior pituitary prolactin-secreting cells (lactotrophs), suggesting that another cell type within the isolated anterior pituitary unit relays the photoperiodic information to the lactotrophs. Since melatonin receptor expression is limited to the PT region of the anterior pituitary, the most logical inference is that it is these PT

cells that relay the effects of photoperiod in the HPD ram model. This led to the hypothesis that the PT secretes a molecule, termed by some *tuberalin*,[173,174] that acts in a paracrine manner to control the activity of PD lactotrophs. In vitro coculture experiments with sheep, hamster, or bovine cell/tissue culture preparations provide some evidence for the existence of a PT-derived prolactin releasing factor[175–178] and also for changes in the level of the production of this factor as a function of seasonal state.[177,178] More recently it has been suggested that this factor may be a Substance P/Tachykinin gene product based on transcriptomic and immunohistochemical analysis of the PT, as well as the in vitro Prl releasing activity of this family of peptide signals.[179,180] Additionally one report suggests a link between PT synthesis of endocannabinoids and PD function in rodents.[181]

The concept that the PT acts as a major relay in the pathway linking photoperiod to seasonal physiology was given considerable impetus by experiments demonstrating melatonin-dependent photoperiodic encoding of clock gene expression in PT of rodents and ungulates.[59–62,182–184] Initial reports described an effect of photoperiod on the amplitude of *Per1* gene expression in hamsters, which could be overridden by pinealectomy or melatonin injection. Subsequently, it emerged that melatonin exerted dual evening and morning effects on rhythmical gene expression, the former being exemplified by induction of *Cry1* by melatonin,[62,183,185] while the latter is characterized by *per1* induction due to melatonin withdrawal. Because PER and CRY proteins form transcriptionally repressive complexes,[186] it was proposed that these effects on PT clock gene expression rhythms might constitute a "coincidence timing" mechanism whereby the photoperiodically variable interval (the coincidence) between the onset and offset of the melatonin signal, and hence the presence of PER/CRY protein, allowed decoding of the photoperiodic message as a change in PT transcription.[62] Experiments in sheep using acute day length extension demonstrated that acute melatonin-dependent effects on the CRY-PER coincidence preceded changes in prolactin secretion but left open the downstream mechanisms through which such effects could be coupled.[183]

The principal secretory cells in the PT have been referred to as "PT-specific cells",[173,187] and are characterized by a much lower density of secretory granules than is seen in the classical granular endocrine cells of the anterior pituitary. They nonetheless show cytology consistent with active secretory function.[188] These cells are of particular interest since they appear to be the major melatonin-responsive cell type in the ovine PT.[136] Whether these cells are the origin of a prolactin releasing factor has yet to be demonstrated, but there is increasing evidence that PT-specific cells represent a thyrotropic cell lineage, distinctive from that seen in the PD. In rodents and in ungulates, RNA expression for both the α and β subunits of thyrotropin (αGSU & TSHβ, respectively) are present in PT-specific cells, and undergo photoperiod-dependent changes in expression (Figure 34.13). [158,189–194] Immunoreactivity for the protein products of these genes has also been observed, but generally with a much weaker signal than is seen in PD endocrine thryotrophs, possibly due to the lack of granular storage in PT-specific cells. While there is no evidence linking the thyrotropic aspect of PT-specific cells to prolactin secretion, PT TSH production is now strongly implicated in seasonal reproductive control. Moreover, recent work provides a mechanism linking the effects of melatonin on clock gene expression in the PT to photoperiodic changes in TSH expression.[114,194] These developments are further discussed later in this chapter.

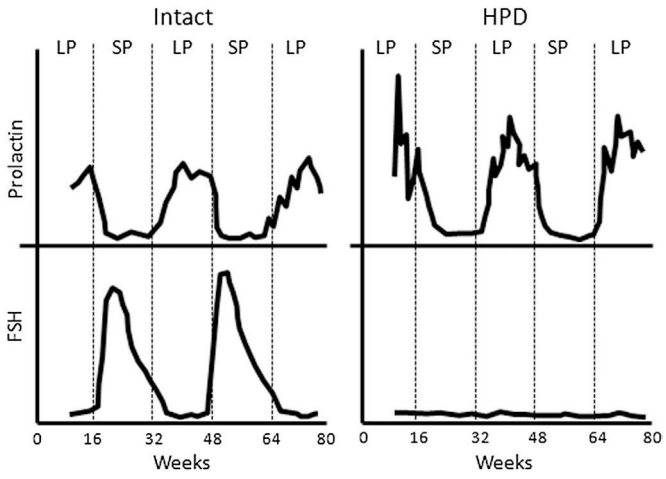

FIGURE 34.12 Persistent photoperiodic control of prolactin secretion in hypothalamo-pituitary disconnected (HPD) Soay rams. Intact and HPD rams were held on alternating photoperiod cycles of 16 weeks 16 h light/day (LP) and 8 h light/day (SP). In intact animals transfer to SP suppresses prolactin secretion and activates FSH secretion. Remarkably, while HPD abolishes control of FSH, the seasonal photoperiodic control of prolactin remains very similar to that seen in intact animals. See text for details. *Source: Based on data in Ref. 172.*

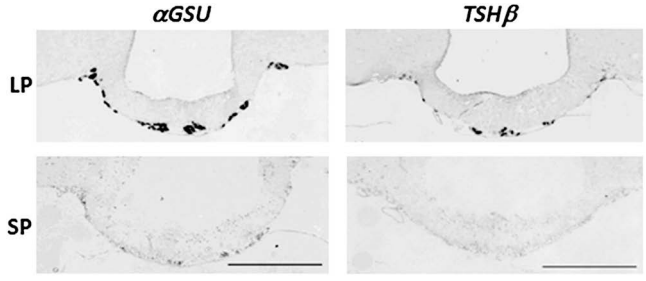

FIGURE 34.13 Photoperiodic variation in α-glycoprotein–subunit (*αGSU*) and thyroid stimulating hormone β–subunit (*TSHβ*) gene expression in the pars tuberalis of the European hamster. Under long photoperiod (LP), high levels of expression of *αGSU* and *TSHβ* mRNA are observed. Under short photoperiod (SP), expression of both genes is dramatically reduced. Scale bar = 200 μm *Source: Adapted from Ref. 189.*

Hypothalamic Lesions and Implants and a "Two Site" Model for Seasonal Reproductive Control

The HPD result linking the PT to prolactin secretion led to what has been described as a "two site" model for seasonal neuroendocrine actions of melatonin.[195] According to this model, while prolactin regulation is PT dependent, the control of seasonal gonadotropin secretion involves an additional site of melatonin action located not in the PT but in the mediobasal hypothalamus. This conclusion is underpinned by a combination of site-specific lesioning and microimplantation studies performed in seasonal hamster species and in sheep. In Syrian hamsters, lesions in the mediobasal hypothalamus region corresponding to IMEL binding sites abolished melatonin-dependent testicular regression.[196,197] In Djungarian hamsters, melatonin infusion in the region of the SCN or SCN lesion altered the testicular response to photoperiod.[198,199] In sheep, melatonin implants placed in the ventromedial hypothalamus or premammillary arcuate regions, but not in PT, effectively abolished photoperiodic synchronization of seasonal breeding, apparently consistent with the presence of IMEL binding sites in these regions.[200–203] These findings have led to the hypothesis that melatonin may act at several, species-dependent hypothalamic sites to exert its effect on the reproductive axis.

The interpretation of microimplant and lesioning experiments outlined herein is hampered by difficulties in excluding actions through the neighboring PT. In the case of melatonin microimplants, Malpaux and colleagues presented evidence based on IMEL implants that drug delivery did not reach the neighboring PT,[204] but given the picomolar affinity of melatonin receptors it is difficult to exclude this possibility beyond doubt. In the case of lesioning experiments, the scenario that lesions simply remove a paracrine target of the PT from the regulatory circuit is difficult to exclude without more concrete data on the pathways involved.

THE PT AND THYROID HORMONE-DEPENDENT CONTROL OF SEASONAL BREEDING

The role of the thyroid gland in the control of seasonal breeding cycles has been suspected since the demonstration that thyroidectomy results in persistent breeding in starlings.[205] Follett and colleagues subsequently observed similar effects in sheep with the spring transition to anestrus, but not the autumn onset of the breeding season, blocked by thyroidectomy.[16] Subsequently, Karsch and colleagues thoroughly characterized the requirement for thyroid hormone in the sheep and demonstrated that the effect of thyroidectomy could be reversed by treatment with thyroxine (T4) in a sensitive window from spring until the mid-summer[206–211] (Figure 34.14). A similar, albeit less detailed, picture has also emerged from studies in seasonal rodent species. In Djungarian hamsters, chemically induced hypothyroidism, achieved by addition of thiourea to the drinking water, advanced the reactivation of the gonadal axis following extended exposure to short photoperiod.[212] Conversely, triiodothyronine (T3) administration blocks seasonal reproductive inhibition and onset of winter physiology in response to short photoperiod in Djungarian (Figure 34.15(B)), and Siberian hamsters.[214–216]

In both rodents and sheep, it has become clear that these effects of thyroid hormone on seasonal breeding rhythms are mediated through sites in the hypothalamus. In the sheep, effects of thyroid hormone microimplants are most effective in the premammillary hypothalamus (PMH), while a lower proportion of implants placed in the ventromedial preoptic area (vmPOA) also were able to induce seasonal anestrus[213,217] (Figure 34.15(A)). In hamsters, mediobasal hypothalamic implants of T3 prevent seasonal anestrus in response to short day exposure[214] (Figure 34.15(B)).

Hypothalamic Deiodinase Mediation of Seasonal Reproductive Role of Thyroid Hormone

A framework for the conceptual synthesis of these findings has emerged with the demonstration that changes in hypothalamic metabolism of thyroid hormone are a primary response to photoperiodic cues in seasonal mammals and birds. The key insight came from a transcriptomic analysis in Japanese quail (*Coturnix japonica*) in a study designed to identify initial hypothalamic changes in gene expression preceding reproductive activation in response to long day exposure.[218] This revealed a rapid induction of type 2 thyroid hormone deiodinase (DIO2) in the first few hours following day length extension. DIO2 is an outer ring thyroid hormone deiodinase, which converts T4 to the more biologically active form T3[219] (Figure 34.16(A)). In the quail, photoperiodic induction of *Dio2* gene expression is associated with a concomitant rise in hypothalamic T3 content, and pharmacological blockade of hypothalamic DIO2 activity by central administration of iopanoic acid prevented photoperiodic activation of the gonadal axis.[218]

Subsequent studies demonstrated that the inductive effect of exposure to long photoperiod on *Dio2* expression also occurred in seasonal rodents[221–226] and in sheep[193] (Figure 34.16(B)). Increased *Dio2* expression was also observed in the median eminence, and in the arcuate or ependymal layer, in goats exposed to artificial long photoperiod.[227] In mammals, this

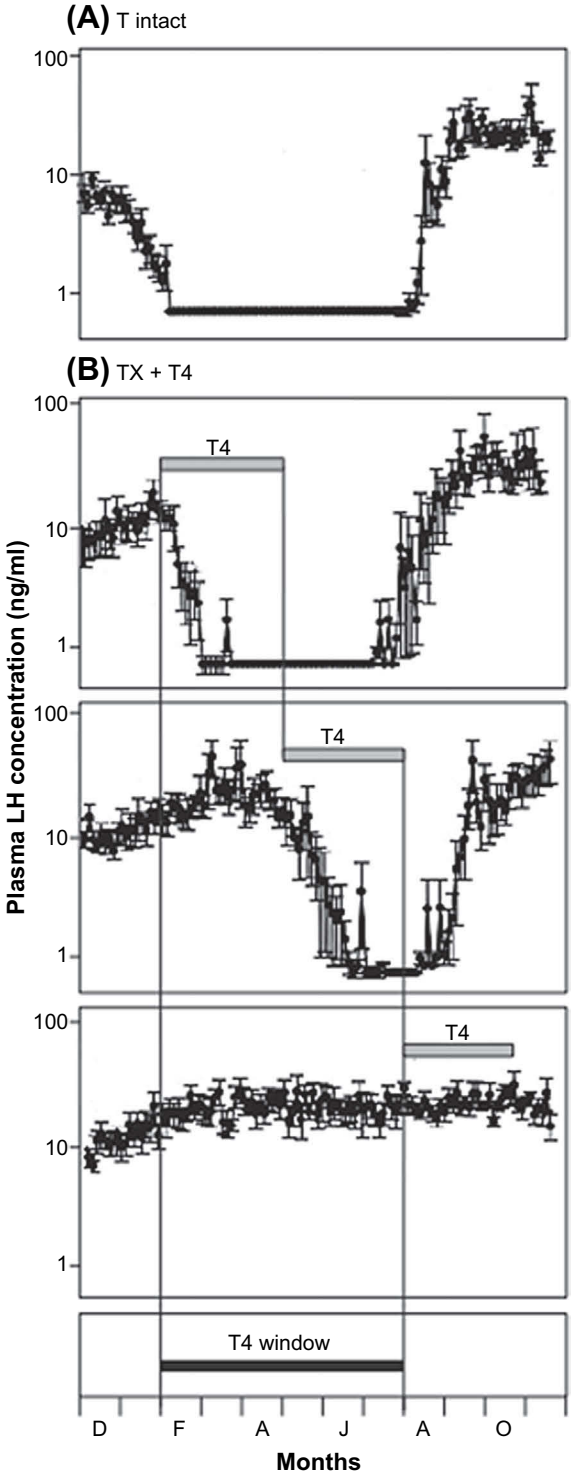

FIGURE 34.14 **The role of thyroid hormone in seasonal breeding cycles in the sheep.** (A) The normal seasonal profile of luteinizing hormone (LH) secretion in estradiol-treated ovariectomized ewes comprises a phase of elevated expression commencing in the autumn and continuing until early the following spring, when a pronounced decline occurs concurrent with the nonbreeding season (N.B. data are plotted on a log scale, and levels during the summer months are below the sensitivity limit of the assay). (B) In thyroidectomized (TX) ewes, thyroxine (T4) replacement permits the seasonal shut down of reproductive function, provided T4 is given in the spring–mid-summer months. Delaying T4 replacement until the late summer–autumn months results in seasonal anoestrous being abolished. Hence a sensitive spring–summer window for T4 actions is defined. *Source: Adapted from Ref. 211.*

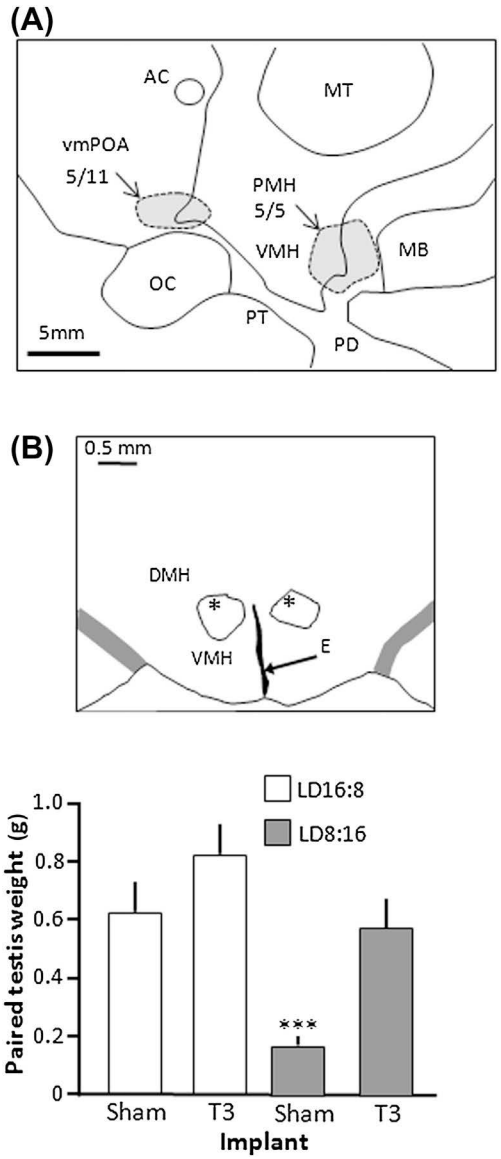

FIGURE 34.15 **Anatomical localization of the seasonal reproductive effects of TH.** (A) In the sheep, T4 microimplants placed in the basal hypothalamus of THX ewes are sufficient to bring about seasonal breeding suppression during the spring. The diagram shows hypothalamic locations in which implants were maximally effective. Abbreviations: AC anterior commissure, MB mammillary body, MT medial thalamus, OC optic chiasm, PD pars distalis, PMH premammillary hypothalamus, PT pars tuberalis, VMH ventromedial hypothalamus, vmPOA ventromedial preoptic area. *(Source: Redrawn from Ref. 213.)* (B) In the Djungarian hamster, T_3-releasing microimplants placed in the mediobasal hypothalamus override the inhibitory effects of short photoperiod on testis size. Asterisks in the upper panel show the approximate location of bilaterally placed microimplants. ***Significantly reduced testis weight compared to LD16:8 sham implant controls ($P<0.001$). *Source: Redrawn from Ref. 214.*

photoperiodic control of *Dio2* appears to be melatonin dependent.[222,223] Furthermore it has become clear that additional genes linked to thyroid hormone metabolism are also photoperiodically regulated. In particular, opposite to *Dio2*, hypothalamic expression of type 3 deiodinase (*Dio3*) appears to be induced by exposure to declining photoperiod[214,220,224,228] (Figure 34.16(B)).

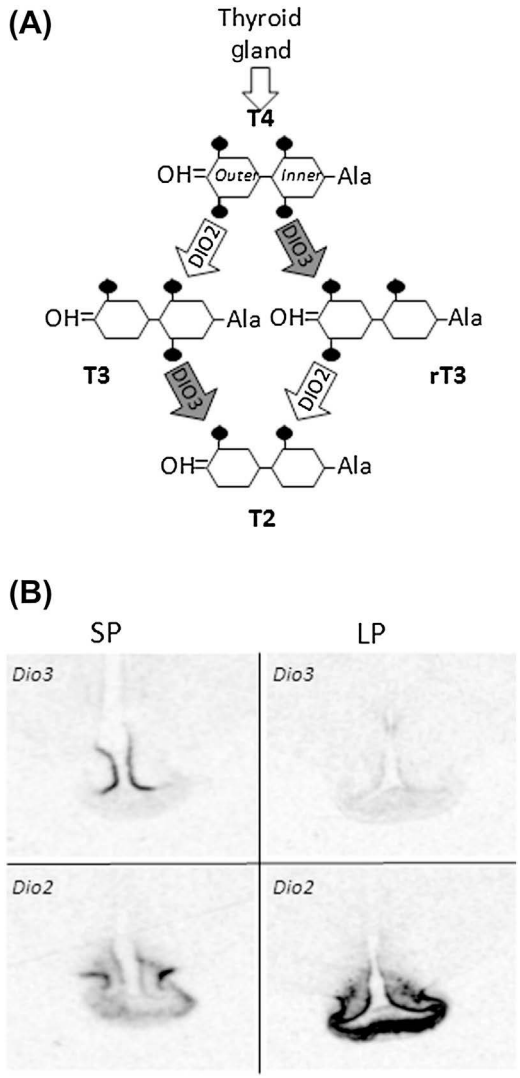

FIGURE 34.16 Thyroid hormone deiodination in the hypothalamus of a seasonal breeder. (A) Enzymatic pathways leading to the conversion of thyroxine (T4) produced by the thyroid gland into either triiodothyronine (T3) by outer ring deiodination catalyzed by type 2 deiodinase (DIO2), or reverse T3 (rT3) by inner ring deiondination catalyzed by type 3 deiodinase (DIO3). The former, activating process leads to increased thyroid hormone actions through nuclear thyroid hormone receptors; the latter is an inactivating process. Further metabolism of T3 or rT3 is possible, leading to diiodothyronine (T2) production, which has no known signaling activity. (B) Reciprocal switching between states of high *Dio2*/low *Dio3* expression and vice versa, through photoperiodic control. Images are autoradiograms of radioactive in situ hybridization histochemistry for *Dio2* or *Dio3* in coronal hypothalamic sections from sheep that were held on either 8 h light/24 h (SP) or 16 h light/24 h (LP) for 4–8 weeks prior to sacrifice. Note strong labeling in the mediobasal hypothalamic area under LP for *Dio2*, but under SP for *Dio3*; note also that *Dio2* labeling extends into surrounding hypothalamic tissue, while *Dio3* labeling is confined to the ependymal zone immediately surrounding the third ventricle. *Source: Adapted from Ref. 220.*

DIO3 is an inner ring deiodinase, converting T4 to reverse T3 (rT3), which appears to be biologically inactive. Recently, monocarboxylate transporter 8 (*Mct8*) gene expression in the Djungarian hamster mediobasal hypothalamus has also been shown to be increased in short photoperiod, similar to *Dio3*[229]; *Mct8* appears to be involved in controlling thyroid hormone uptake into the central nervous system,[230] implying a global photoperiodic regulation of pathways linked to hypothalamic thyroid status.

Although there appear to be differences in the details (for example, in Djungarian hamsters *Dio3* but not *Dio2* is sensitive to short day exposure[214] whereas both genes are photoperiod sensitive in the European hamster, Soay sheep, and the common vole (*Microtus arvalis*)[220,224,225]), the general picture is that long day exposure promotes a euthyroid state in the hypothalamus (increased T4 uptake and conversion to T3), while short day exposure has the opposite effect, promoting local hypothyroidism. Importantly, this general picture is independent of whether species are short or long day breeders. Additionally, innately timed changes in expression of *Dio2* and *Dio3* are associated with the onset of photorefractoriness,[220,229] suggesting that spontaneous long-term changes in hypothalamic thyroid metabolism may generate circannual reproductive rhythms.

The expression of deiodinases and of *Mct8* is highly localized in the hypothalamus, being confined to the ependymal zone surrounding the ventral portion of the third ventricle. The ependymal layer in this region contains a mixture of typically cuboidal ependymal cells and specialized glial cells known as tanycytes.[231] Unlike the cuboidal ependymal cells, tanycytes have cytosolic projections into the parenchyma of the surrounding basal hypothalamus, as well as down into the median eminence. While definitive evidence is lacking, and expression in neighboring glial cells cannot be excluded, the patterns of *Dio2*, *Dio3*, and *Mct8* expression are all consistent with enriched expression in the third ventricle tanycyte cell population. The biological role of tanycytes has been much discussed (see Refs 231–233). They appear critical in the estrous regulation of reproduction in female rodents.[234] In addition to being a specific site of deiodinase expression, tanycyte structure undergoes a marked melatonin-driven remodeling.[235] Thus, these cells are now receiving renewed attention in the context of seasonal reproduction.

Relationship between the PT and Deiodinase Expression in Tanycytes

Recently it has become clear that in mammals, melatonin-dependent production of TSH by the PT mediates photoperiodic regulation of deiodinase expression in the hypothalamic ependymal zone[158,193,236] (Figure 34.17), challenging the two-site model for melatonin's role in seasonal breeding. It has long been known that thyrotropic cells comprise a significant proportion of the endocrine cell population present in the PT, that these cells

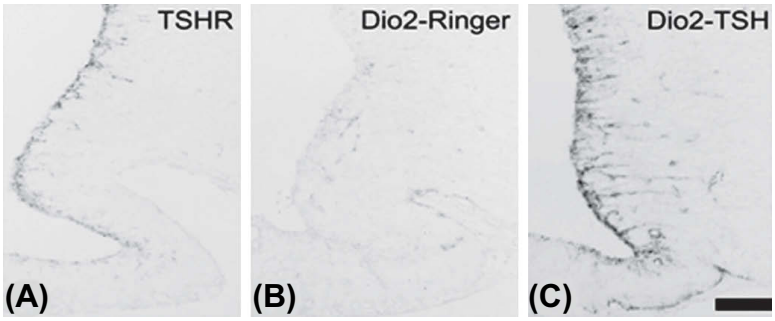

FIGURE 34.17 Central infusion of TSH promotes *Dio2* expression in tanycytes surrounding the base of the third ventricle. In the male Djungarian hamster, tanycytes express TSH receptors (TSHR, left panel) and acute intracerebroventricular infusion of 1 mIU TSH (right panel), but not of Ringer solution (middle panel) induces *Dio2* mRNA expression within 4 h in short day-adapted animals. Scale bar = 100 μM
Source: Adapted from Ref. 237.

express MT1, and that levels of PT TSH expression vary with seasonal state in seasonal rodent species[189,238–240] (Figure 34.13). These cells constitute a distinct population developmentally from those found in the PD and appear not to express receptors for thyrotropin-releasing hormone,[191] placing them outside of the classical thyrotropic regulatory axis.

Interest in PT-derived TSH as a regulator of seasonal reproductive physiology was reinvigorated with the finding that expression of TSH receptor (TSHR) RNA in the basal hypothalamus shows a remarkable overlap with that for *Dio2*/*Dio3*.[158,193,224,236] Exposure to long photoperiod causes a rapid induction of *Tshβ* RNA expression in the PT in sheep,[193] hamster[189] (see Figure 34.13), and in melatonin-proficient lines of mice.[158] Since *TSHβ* subunit expression is rate limiting for synthesis of TSH,[241] these observations suggested that PT TSH might act in a paracrine manner on neighboring ependymal cells to control deiodinase expression. Direct evidence for this interpretation is provided by the demonstration that central TSH infusion induces *Dio2* expression in SP-acclimated sheep[193] and hamster[237] (Figure 34.17), and by the finding that *Tshr* knockout blocks photoperiodic induction of *Dio2* in melatonin-competent mice.[158]

Remarkably, PT-derived TSH appears to be the key relay between photoperiod and deiodinases in birds as well as mammals, despite melatonin apparently not being required for seasonal control of reproduction in birds.[236,242] Rather, deep brain photoreceptors, with opsin-like sensitivity characteristics located in paraventricular sites within the diencephalon, are proposed to project to the avian PT and control its function.[243,244]

Follow-up studies addressing the issue of how melatonin regulates TSH production in the PT highlight the role of the transcriptional coactivator, eyes absent 3 (EYA3), as an upstream signal controlling *Tshβ* transcription.[114,180,194] EYA3 acts in concert with other members of a network of transcription factors, coactivators, and corepressors associated with eye, pituitary, and limb development.[245] In the PT, EYA3 potentiates the transcriptional activating effects of thyrotroph embryonic factor (TEF) to drive *Tshβ* expression. *Eya3* transcription is under circadian control, with a phase of peak expression occurring approximately 12 h after melatonin onset in the evening. Additionally, the amplitude of peak *Eya3* expression is directly repressed by melatonin.[114,194] These two effects appear to combine to give day length dependent *Eya3* expression in the PT, with long days/short melatonin signals favoring increased *Eya3* expression due to absence of melatonin at the phase of peak *Eya3* expression some 12 h after dark onset (Figure 34.18). This in turn leads to photoperiodic control of *TSHβ* expression[114,194] (Figure 34.18). Promoter analysis of the *Eya3* gene shows that it is under circadian control through E-box response elements, suggesting that *Eya3* regulation is the key link between the effects of melatonin on circadian clock gene expression discussed above and seasonal photoperiodic readout in the PT.[62,114,180,194]

Recent data from sheep indicate that the innately timed return to breeding condition with extended exposure to long photoperiod (i.e., long day photorefractoriness), involves parallel declines in brain deiodinase and PT *TSHβ* gene expression.[220,222] This spontaneous decline may account for the limited time window during which a T4 implant can elicit termination of the breeding season on TX ewes[211] (Figure 34.14). Conversely, in the sheep, the spontaneous return to anestrus after extended short photoperiod exposure (i.e., short day photorefractoriness) involves changes in deiodinase expression, without any measurable effect on PT *TSHβ* expression,[220] and earlier reports in hamsters indicate that short day photorefractoriness develops without observable increases in *Dio2* expression.[222] These findings suggest that long-term timing of seasonal breeding is generated at more than one site in the neuroendocrine system, but leave open the fundamental question of how these long-term timing effects are generated.

HYPOTHALAMIC NEURAL PATHWAYS INVOLVED IN NEUROENDOCRINE CONTROL OF SEASONAL BREEDING—THE ROLE OF RF-AMIDE SIGNALING PATHWAYS

GnRH neurons in the rostral hypothalamus (preoptic area and organum vasculosum of lamina terminalis) represent the final common pathway in the neural

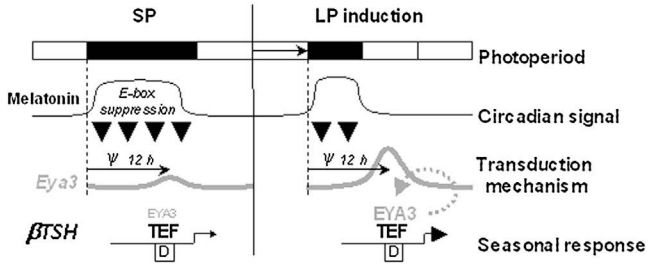

FIGURE 34.18 Model for photoperiodic control of Eya3 and TSHβ expression in the pars tuberalis. The model proposes that melatonin exerts a direct suppressive effect on *Eya3* expression and an indirect effect via circadian gene oscillations in the pars tuberalis. The phase of the circadian rhythm of *Eya3* expression *relative* to the rhythmical presence of melatonin (Ψ) is critical for determining whether a strong peak of *Eya3* expression occurs. Independent of day length, *Eya3* peaks some 12h after dark (melatonin onset). This means that under short days (SP, left panel), the peak occurs during the night, while the melatonin level is high and exerting a suppressive effect, and so the peak is small. Contrastingly, under long days (LP, right panel), the *Eya3* peak occurs the following morning when the melatonin level is minimal, and so the peak is large. This classic "external coincidence timer" mechanism limits EYA3/TEF synergism and hence elevated levels of *TSHβ* expression to long days, controlling the onset of a summer phenotype. *Source: Modified from Ref. 114.*

regulation of the hypothalamo-pituitary-gonadal axis (see Chapter 11). GnRH neurons project to the median eminence where they release GnRH into the portal blood to stimulate the synthesis and release of the pituitary gonadotropins luteinizing (LH) and follicle-stimulating (FSH) hormones, which, in turn, control gonadal growth and activity. Early experiments have demonstrated that in most species the number of GnRH neurons and the content of GnRH immunoreactivity do not change during seasonal reproductive quiescence, despite a marked decrease in gonadotropin secretion and gonadal activity.[246] Rather, the release of GnRH in the portal blood shows remarkable photoperiodic variation, indicating that melatonin acts to control the neurosecretory activity of GnRH neurons.[247,248] As discussed in Chapter 27, a marked increase in sensitivity to the negative feedback effects of gonadal steroids appears to be a key feature of seasonal reproductive quiescence in sheep. A similar photoperiod-controlled change in response to testosterone negative feedback has been observed in male hamsters.[249] This implies that the key downstream consequence of inhibitory photoperiod is a potentiation of steroid negative feedback effects on GnRH neurons in seasonal breeders. Since there is no evidence that GnRH neurons are directly sensitive to sex steroid feedback,[250,251] current attention focuses on interneuronal pathways linking melatonin and sex steroid sensitivity to the GnRH pulse generator.

In the sheep, evidence implicates the A15 group of dopaminergic (DA) neurons in the inhibition of GnRH secretion during seasonal anestrus, and these DA neurons are activated by estrogen indicating that they may mediate estrogen negative feedback[252] (see Chapter 27). Consistent with this hypothesis, short photoperiod- or melatonin-induced reproductive activation is associated with a decline in tyroxine hydroxylase (the rate limiting enzyme in DA synthesis) activity in the DA terminals of the median eminence.[253] Although other studies support a putative role of DA in the photoperiodic control of reproduction in horses,[254,255] at the present time, there is no evidence linking DA signaling to photoperiodic shifts in gonadotropin secretion in seasonal rodents, and recent attention has instead focused on the RF-amide (arginine-phenylalanine-amide) family of peptides. In particular, Kp and RF-amide related peptide-3 (RFRP-3) have emerged as potent regulators of GnRH neuronal activity.

Kisspeptin in the Arcuate Nucleus is a Gate-Keeper of Seasonal Reproduction

The *Kiss1* gene[256] gives rise to a Kp precursor peptide that may be cleaved to give fragments ranging in size from 10 to 54 amino acids (Kp10 and Kp54, respectively), with similar N-terminal decapeptide characteristics. These act as ligands for the G protein–coupled Kiss1 receptor (Kiss1r, previously known as GPR54). In 2003, it was shown that loss of function mutations of the *Kiss1r* in humans and mice prevent pubertal development and cause infertility.[257–259] This remarkable finding spurred great interest in the role of Kp in reproductive function.[260] In mammals, low doses of central Kp10 or Kp54 have a powerful stimulatory effect on GnRH neuron activity, gonadotropin secretion, and sex steroid production. Kp neurons are found mainly in two hypothalamic areas in rodents, the ARC and a more rostral area, the anteroventral periventricular nucleus (AVPV), while in the sheep they are found within the ARC as well as in the preoptic area.[192,261,262] In rodents, the AVPV Kp neuronal population is much larger in females than in males, in line with the pivotal role of AVPV Kp in the control of the preovulatory LH surge.[263,264] ARC Kp neurons also co-express neurokinin B and dynorphin, and are now therefore referred to as KNDy neurons.[265] Kp neurons project to areas where GnRH neurons are located, and over 90% of GnRH neurons express Kiss1r.[266,267] Additionally, Kp fibers extend toward the median eminence where the peptide acts on GnRH nerve terminals to regulate GnRH release.[268] Strikingly, Kp neurons are the main target for the positive (in AVPV/preoptic area) and negative (in ARC) central feedback effects of sex steroids, underlying their pivotal role in the tightly controlled positive/negative feedback loops of the reproductive axis.[262,269–271]

The pivotal role of Kp in the control of pubertal reproductive activation suggested that Kp neurons might also be involved in seasonal reproductive

control. Consistent with this hypothesis, seasonal mammals exhibit significant photoperiodic variation in Kp expression, albeit with marked differences between species.[272] In the Syrian hamster, Kp expression increases in the long day breeding season, both in the AVPV and the ARC, and this appears to be a melatonin-dependent response[262,271] (Figure 34.19(A)). Chronic administration of Kp, either centrally or peripherally, fully reactivates the reproductive activity of male Syrian hamsters exposed to short photoperiod,[271,274] implying a key role for seasonal changes in Kp expression for control of the reproductive axis (Figure 34.19(A), lower panel). A similar picture has emerged from studies in the short day breeding sheep: Kp expression is increased within the caudal part of the ARC upon exposure to short photoperiod[192,261,275] (Figure 34.19(B)), and Kp infusion drives reproductive activation in ewes kept in photo-inhibitory conditions[273,276] (Figure 34.19(B)). Melatonin, however, does not act directly on Kp neurons because, in the Syrian hamster, the ARC region lacks IMEL binding sites,[121,122,277] and in the sheep, Kp neurons are devoid of melatonin receptors.[278]

While the data in Syrian hamsters and sheep both suggest that ARC changes in Kp expression mediate changes in seasonal reproductive activation, the picture is complicated somewhat by data showing that, in the Djungarian hamster, long photoperiod suppresses ARC Kp expression.[279,280] Potentially this discrepancy might be due to superimposed effects of testosterone negative feedback on Kp expression in these reproductively active hamsters. In the short photoperiod–adapted Syrian and Djungarian male hamsters, increasing circulating testosterone levels by chronic implants induces a marked inhibition of Kp expression in the ARC but a stimulation of expression in the AVPV.[262,271,280] In the sheep, the estrogen-sensitive DA neurons project to the Kp neurons to inhibit their activity in anestrus.[281] The effects of steroid feedback on Kp expression are complex, and the resulting levels of Kp may be different among species. Additionally, metabolic parameters, notably leptin, also influence Kp expression,[282–285] adding another level of complexity in the seasonal context. Clearly further work is required to resolve the complex interplay between these factors and photoperiodic modulation of Kp signaling networks.

RFRP Neurons in the Mediobasal Hypothalamus May Mediate Melatonin Action on Seasonal Reproduction

Other peptides of the RF-amide family, characterized by a common C-terminal LPXRFamide (X = L or Q) motif were shown to regulate reproductive activity in birds and mammals (for reviews, see Refs 286–288). In the quail, one such peptide acts directly at the pituitary to inhibit gonadotropin release and is accordingly named gonadotropin-inhibitory hormone (GnIH).[289] Additionally, GnIH expression in the avian paraventricular hypothalamus is increased by a direct activation of Mel1c receptors.[290] In mammals, the *RF-amide related peptide* gene (*Rfrp*), the mammalian ortholog of *GnIH*, is expressed in neurons located in the mediobasal hypothalamus, and it encodes a precursor that produces two peptides, RFRP-1 and RFRP-3.[291,292]

RFRP neurons project to multiple brain regions including the preoptic area, the ARC, the lateral septum, the anterior hypothalamus, and the bed nucleus of the stria terminalis. Notably, RFRP-immunoreactive fibers make apparent contacts with 20–40% of GnRH neurons in rodents and sheep[292–295] and a subpopulation of Kp neurons,[296] suggesting that RFRPs act centrally to control the reproductive axis. The first functional studies in mammals reported that central or peripheral administration of RFRP-3 reduces LH secretion in male and female rats and ewes.[292,297–299]

Several studies have explored photoperiodic regulation of RFRP expression in mammals. In the Syrian hamsters, levels of *Rfrp* mRNA and RFRP are markedly downregulated in short as compared to long day conditions with no significant daily variation in both photoperiods[300] (Figure 34.20(A)). A similar short day inhibition of RFRP expression was observed in the Djungarian[295,300] and European hamsters,[288] the jerboa,[302] and, to a lesser extent, in the sheep,[261,303] suggesting common regulatory mechanisms. In male Syrian and Djungarian hamsters, the short day inhibition of RFRP expression is driven by melatonin and independent of the photoperiodic variation in the circulating levels of sex steroids.[295,300]

The role of RFRP in reproductive regulation in mammals seems to vary with species, and with annual phase of the breeding season. In short day breeding sheep, RFRP peptides have been described as having an opposed role to that of Kp, suppressing gonadotropic activity in the summer,[298,304] although a recent study could not replicate this observation.[305] Contrastingly, RFRP-3 activates the gonadotropic axis in male Syrian and Djungarian hamsters. In the Syrian hamster, central injection of RFRP-3 in long photoperiod–acclimated animals activates GnRH neurons and increases gonadotropin and testosterone secretion[301] (Figure 34.20(B)). In the same species, a 5-week central infusion of a low dose of RFRP-3 was able to restore testicular function to levels comparable to those observed in long day–acclimated hamsters.[301] Importantly, this RFRP-3-induced reactivation of reproductive function was associated with a significant increase in *Kiss1* expression the ARC. In the male Djungarian hamster, central infusion of RFRP-3 increases LH secretion in short day conditions, whereas it is inhibitory in long day conditions.[295] Of note, however, another study reported that RFRP-3 administration in ovariectomized female

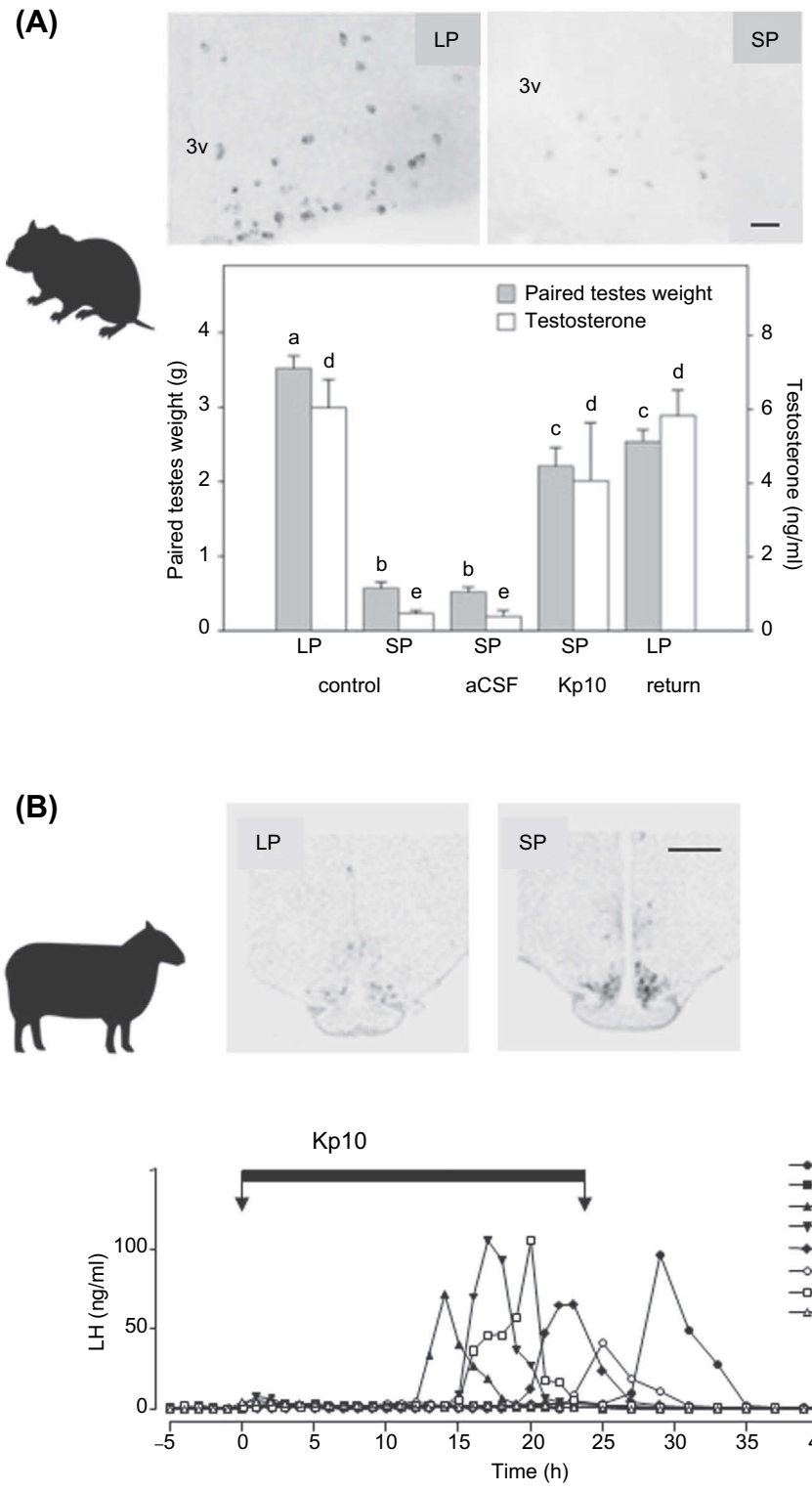

FIGURE 34.19 Kisspeptin (Kp) plays a key role in photoperiodic regulation of reproduction. (A) In the male Syrian hamster; *Upper panel*, the number of *Kiss1* mRNA–expressing neurons in the ARC is significantly increased in long photoperiod (LP) exposed sexually active animals as compared to short photoperiod (SP) adapted animals; scale bar = 100 µM; *(Source: Modified from Ref. 262.) Lower panel*, chronic (4 weeks) intracerebroventricular infusion of Kisspeptin 10 (Kp10, 1 mM, 0.25 µl/h) partially restores testicular function in short photoperiod (SP)–adapted male Syrian hamsters. Note that Kp10-induced testicular activity is similar to that reached in hamsters returned to LP for 4 weeks (LP return) (control = non-infused animals; aCSF = animals infused with artificial CSF). Bars with differing letters differ significantly ($p < 0.05$); *(Source: Adapted from Ref. 271.)* (B) In the sheep; *Upper panel*, autoradiographs showing elevated *Kiss-1* expression at the level of the arcuate nucleus of female Soay sheep kept under short photoperiod compared to ewes on long photoperiod (SP/LP, respectively); Scale bar = 1 mm; *(Source: Adapted from Ref. 192.) Lower panel*, patterns of secretion of luteinizing hormone (LH) in individual anestrous ewes infused with Kp10 (15.2 nmol/h) for 24 h (infusion period is represented by the black bars and arrows). Surges of LH secretion (in response to an increase in estradiol secretion stimulated by a rise in tonic LH) occurred between 19 and 34 h after the start of the infusion of Kp10 *Source: Adapted from Ref. 273.*

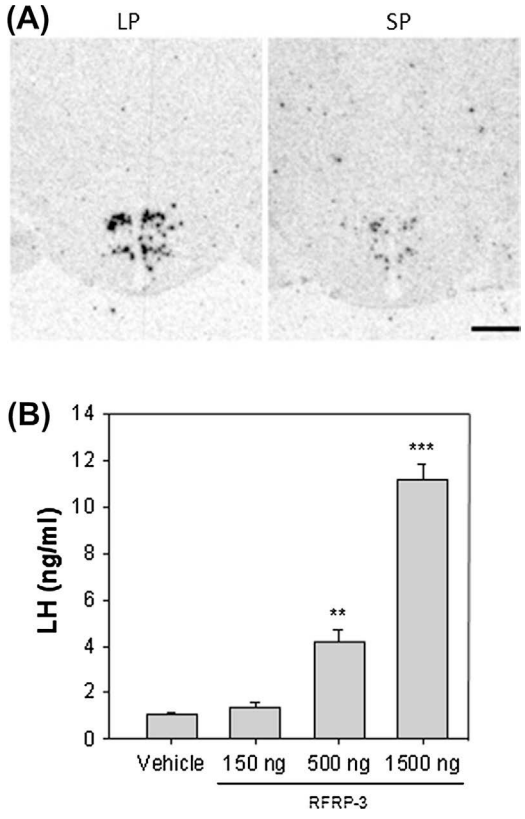

FIGURE 34.20 Photoperiodic regulation and role of RFRP 3 in the male Syrian hamster. (A) Autoradiographs showing that Rfrp mRNA levels in the dorsomedial/ventromedial hypothalamus are markedly reduced in short photoperiod (SP) as compared to long photoperiod (LP) conditions; Scale bar = 1 mm; (*Source: Adapted from Ref. 300.*) (B) Acute intracerebroventricular administration of RFRP 3 induces a dose-dependent increase in LH secretion within 30 min. Data are mean ± SEM ($n = 7$ per group) of circulating LH value examined 30 min after the central injection (***, $P < 0.001$) *Source: Adapted from Ref. 301.*

Syrian hamsters reduces LH secretion.[292] Taken together, these findings indicate that while short photoperiod exposure inhibits RFRP expression in the mediobasal hypothalamus of various seasonal breeders, the downstream consequences are species and possibly gender specific. This divergence of action possibly reflects differences in pharmacology and cellular sites of action.

Thyrotropin-Dependent Control of Hypothalamic RF-Amide Expression and Reproduction

Recent data indicate that photoperiodic, melatonin-dependent changes in both RFRP and Kp expression are downstream consequences of changes in PT TSH signaling and regulation of deiodinase expression.[237] In short day–acclimated, sexually inactive, male Syrian and Djungarian hamsters, chronic central infusion of TSH fully reactivates their reproductive function. This effect occurs in parallel with an increased expression of *Dio2* in the ependymal zone and a restoration of RFRP expression to long day levels. Interestingly, in both species, the expression of Kp is also restored, although not completely to a long day pattern.[237] These observations implicate RFRP neurons in the downstream hypothalamic response to melatonin-driven changes in TSH/T3. Given that RFRP-3 stimulates reproductive activity in the male Syrian and Djungarian hamsters,[295,301] but has the opposite effect in sheep,[298,304] it is tempting to speculate that the divergent downstream consequences of melatonin-sensitive deiodinase gene expression may be accounted for by species-dependent actions of RFRP-3 on the gonadotropic axis.

HYPOTHESES LINKING HYPOTHALAMIC T3 CONTENT TO HYPOTHALAMIC NEURAL CHANGES DRIVING SEASONAL BREEDING: A PIVOTAL ROLE FOR TANYCYTES?

Two basic conclusions emerge from the foregoing discussion: (1) Deiodinase-dependent changes in hypothalamic thyroid hormone metabolism, which determine T3 levels in the hypothalamus, are crucial for the expression of seasonal changes in reproductive activation in mammals, and (2) RF-amide based neural circuits are seasonally regulated modulators of reproductive activation, providing the interface between gonadal steroid feedback and GnRH pulse generator activity. These appear to be the major upstream and downstream elements for inclusion in the current working model, and from this the question emerges of how T3 availability influences RF-amide–dependent GnRH release (Figure 34.21). Several hypothetical mechanisms may now be considered.

Hypothesis 1: T3 Produced by Tanycytes Influences Hypothalamic Neuronal Excitability

Coppola and colleagues[306] have argued that mitochondrial activity is critical for sustained neuronal activity in hypothalamic NPY/AGRP neurons involved in energy sensing, and that T3 made available by surrounding glial cells (including tanycytes) supports mitochondrial activity by stimulating mitochondrial gene expression (especially uncoupling protein 2). Tanycyte expression of *Dio2* might mediate this effect and favor increased neuronal excitability under long photoperiod. Since tanycytes project widely into the parenchyma, encompassing the ARC, the ventromedial hypothalamus and the median eminence, this mechanism may extend to a wider variety of cell types than that specifically considered by Coppola et al., and could conceivably impact on the behavior of Kp/RFRP neurons linked to seasonal reproductive control.

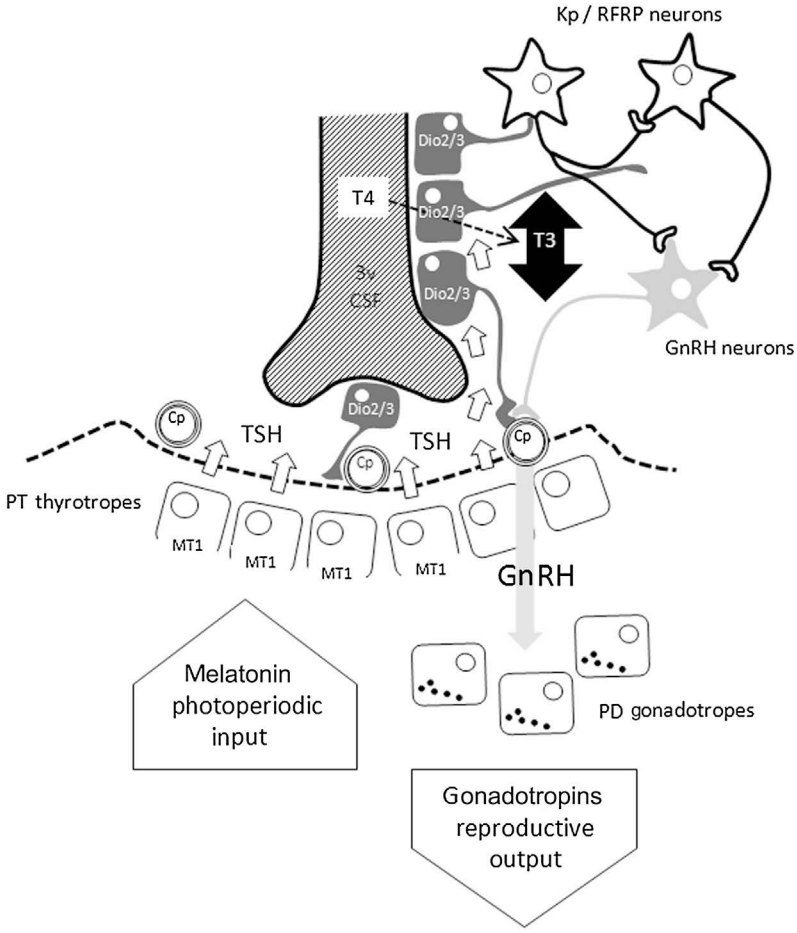

FIGURE 34.21 Working model for the pituitary/hypothalamic network regulating seasonal reproduction in mammals. Photoperiodic input is relayed to the PT via pineal production of melatonin and expression of type 1 melatonin receptors (MT1) in PT cells. Dependent on the duration of the nocturnal melatonin signal, these cells produce high or low levels of TSH, which act on tanycytes surrounding the base of the third ventricle (3v). High levels of TSH promote high levels of *Dio2* expression in tanycytes, while low levels favor *Dio3* expression. These enzymes determine the levels of T3 in the mediobasal hypothalamus. T4 is presumed to reach the ependymal region via the CSF, although supply via brain capillaries (Cp) is not excluded. The resultant photoperiodic modulation of hypothalamic T3 levels in turn affects the activity of the neuropeptidergic neurons governing pulsatile GnRH release. This may include effects of T3 on GnRH nerve terminals, or indirect effects via RF-amide neurons expressing Kp/RFRP. Resultant changes in pulsatile GnRH in turn govern pars distalis (PD) gonadotropin secretion and hence reproductive output.

Hypothesis 2: Tanycytes Modulate Energy Sensing

Central, hypothalamic actions of T3 may be important for the control of thermoregulation.[307] AMPK is a sensor of energy stress, whose activation through AMP accumulation/ATP depletion (the AMP:ATP ratio[308]), slows cell metabolism and lipid biosynthetic pathways. In the hypothalamus, AMPK-dependent changes in lipids are believed to influence neuronal excitability, possibly through effects on ion channel activity or intracellular calcium levels.[309] Hypothalamic T3 downregulates AMPK signaling in hypothalamic energy circuits, and this increases sympathetic thermogenic drive to brown adipose tissue. Interestingly, T3 implants block the capacity to lower body temperature and enter torpor in short day–acclimated Djungarian hamster.[216] Given that the capacity to enter torpor is highly dependent on metabolic status, a strong hypothesis is that this effect of T3 implants is mediated through a shift in AMPK-dependent energy sensing in the hypothalamus. By extension, seasonal shifts in T3-dependent modulation of AMPK sensing could alter the activity of neurons controlling GnRH pulse generator activity.

There is also evidence that tanycytes themselves act as hypothalamic energy sensors, a function consistent with their anatomy and morphology. Recent studies show that tanycytes respond to glucose by an increase in intracellular Ca^{2+} and ATP release.[310,311] Interestingly, a recent study reported that chronic TSH infusion in short day–adapted Djungarian hamster restored the long day phenotype of body weight together with that of reproduction.[237] At this time, the relationship between these energy sensing mechanisms, and photoperiodic influences of TSH and T3 metabolism, remain unclear, but this is likely to remain an active line of enquiry in the next few years.

Hypothesis 3: Tanycytes Support Hypothalamic Neurogenesis

Recent studies suggest the presence of a neurogenic stem cell niche in the basal hypothalamus, and that tanycytes in this niche are neural stem cells.[312,313] Pharmacological[314] or radiological[312] insults to cell division in the tanycyte-containing ependymal layer at the base of the third ventricle produce long-term, persistent changes in appetite and energy balance in mice, suggesting that

neurogenesis is required for maintenance of hypothalamic function. Cell division in this region yields progeny neurons that migrate into neighboring arcuate regions and acquire peptidergic phenotypes (e.g., AGRP+) consistent with their incorporation into circuits involved in metabolic regulation.[312,313] Accordingly, the possibility that neurogenesis-dependent changes in hypothalamic neural circuitry form the basis for long-term cycles of seasonal physiology merits attention.[15] At the time of writing, however, no specific link between the proposed hypothalamic stem cell niche and seasonal physiology has been made, despite several correlative reports of changes in hypothalamic cell proliferation related to seasonal status.[315–319] Given that T3 levels have been linked to neurogenic processes in other brain regions, also involving tanycytes (e.g., Ref. 320), it is tempting to suggest that seasonal changes in T3 availability may drive stem cell–dependent cycles of neural change linked to reproduction.

These three hypotheses for the downstream consequences of seasonal changes in tanycyte function/hypothalamic T3 metabolism are by no means mutually exclusive, and an attractive scenario is that distinct mechanisms operate to bring about rapid "activational" change at one phase of the circannual cycle, while others mediate long-term "preparative" change with delayed consequences months into the future.

CONCLUSION

The establishment of hypothalamic processing of thyroid hormone as the major pathway through which photoperiod synchronizes seasonal cycles of breeding represents a major advance in the last decade. This pathway appears to be conserved across mammals, regardless of the timing of their breeding season, and to rely on melatonin actions through the PT. This pathway does not exclude other routes of melatonin action, but given the explanatory power of the TSH-deiodinase model, we suspect that elucidation of additional pathways will lead to refinement rather than paradigm shifting. This is not to say that there are not a good deal of important unanswered questions in this field; rather, the current model brings several longstanding issues back to the forefront of research interest and should help to formulate new hypotheses. We still do not understand how widely species-divergent phasing of the breeding season emerges from basically conserved regulation of hypothalamic thyroid availability, but the complexity of RF-amide effects in seasonal breeders indicates that downstream signaling networks will be crucial to resolving this conundrum. The fundamental nature of innate long-term timing of seasonal breeding cycles, and other circannual physiology, is another mystery for which the current model suggests new hypotheses, with the role of hypothalamic tanycytes now under the spotlight. Another prospect is extension of progress in laboratory animal models to consider mechanisms in more ecological settings. The interaction between photoperiod and other cues to time seasonal reproduction in mammals, and the influence of ecology on genetic variation in seasonal timing mechanisms, have become very timely problems because of their topicality in relation to climate change. We can now turn to these issues because modern genomic tools will bring molecular physiologists and ecological geneticists together in an unprecedented manner.

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CHAPTER

35

Physiological Mechanisms for the Metabolic Control of Reproduction

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INTRODUCTION

Reproduction, as the biological process whereby organisms produce new organisms of the same kind, is essential for the survival and perpetuation of any given species. Accordingly, reproductive maturation and function are subjected to precise regulatory mechanisms, which impinge at different levels of the hypothalamic–pituitary–gonadal (HPG) axis. As described elsewhere in this book, this neurohormonal system is primarily composed of the hypothalamic factor, gonadotropin-releasing hormone (GnRH); the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH); and gonadal hormones of a steroid and peptidergic nature. These factors are connected by feedforward and feedback regulatory loops, which are organized at early developmental periods and are responsible for the dynamic regulation of the reproductive system during the life span.[1]

The function of the HPG axis is tightly connected to the mechanisms governing other key body systems.[1] Yet, in contrast to other relevant biological functions, such as brain activity and metabolism, pubertal maturation and fertility are dispensable at the individual level. Hence, numerous conditions that disturb organism homeostasis, including different forms of nutritional stress, can result in reproductive perturbations that, depending on the time of presentation, manifest in either the lack of proper pubertal maturation or the loss of reproductive capacity in the adult.[1] The mechanisms for such a *collateral* regulation of the reproductive system are likely multifaceted, but a nidus for this control is located at central levels of the HPG axis, and comprises direct or indirect modification of GnRH neuronal function.[1–5]

It is now well known that the reproductive axis in general and GnRH neuronal function in particular are highly sensitive to the magnitude of body energy reserves and to the modulatory actions of several nutritional and metabolic factors.[1–7] This connection between body weight, as an index of energy reserves, and fertility was probably inferred on the basis of intuitive knowledge as early as the Paleolithic Age, when fecundity icons, globally referred to now as *Venus figurines*, were often represented as overweight women. However, it was not until the 1960s and 1970s that this relationship became formulated on a scientific basis.[6,8] Although the pioneering studies in experimental animals by Kennedy and Mitra set the grounds for this formulation,[9] it was the work in humans of Rose E. Frisch and colleagues, using first epidemiological data and later results from clinical experimentation, that crystallized these concepts into the so-called critical fat mass hypothesis.[10,11] This hypothesis was the first to point out the need to reach a certain threshold of body (fat) mass in order to complete pubertal development and/or sustain an appropriate reproductive function in adulthood. This hypothesis, which stated that body fatness is a better predictor than chronological age for the timing of puberty, provided the biological basis for the switch-off of the reproductive axis in adverse metabolic conditions. Since reproduction, and especially pregnancy and lactation, involve a substantial metabolic drain on the female,[12] suppression of puberty and fertility could be considered a failsafe mechanism to ensure the proper supply of energy substrates to life-sustaining body systems and processes in situations of limited energy resources.

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Admittedly, the Frisch hypothesis had several limitations and was the center of numerous criticisms and strong debate among both clinical and basic scientists.[13–15] It was initially pointed out that weight and height, as used by Frisch and colleagues, were not fully reliable as proxy indices of body fatness. It was also suggested that the numerous exceptions to the fixed threshold dictated by body weight and adipose content, as well as the concurrence of numerous co-variables (e.g., in conditions of very low body weight), which may explain alterations of puberty and fertility per se, rendered this formulation of limited value.[16] It can be added that the work of Frisch did not consider the potential effects of very early metabolic influences, later shown to affect pubertal timing,[17] and did not address the impact of alterations at the other end of the spectrum of metabolic disorders, such as obesity. In fact, obesity and related alterations have warranted considerable contemporary attention because of their epidemic proportions and discernible effects on human puberty and fertility, as revealed by recent studies.[18–21]

Another aspect that was not sufficiently considered in the above studies was the impact of metabolic cues in male reproduction. Indeed, although this was initially neglected, it has been now documented that, through common and specific regulatory pathways, male puberty and reproductive capacity in adulthood are also sensitive to the influence of metabolic signals.[22] The teleological reasons of such a connection may appear less obvious, since male reproduction does not apparently face similar metabolic demands as in the female. Notwithstanding, in numerous species, key aspects of reproductive performance in the male, such as territoriality and dominance, are closely linked to, and dependent on, adequate energy conditions.[4] Yet, it is also obvious that there is a marked sexual dimorphism in some aspects of the metabolic control of reproduction.

In any event, despite its limitations, the work of Frisch and colleagues, together with the previous observations of Kennedy and Mitra on the roles of body weight and food intake in puberty initiation in rats,[9] highlighted the connection between body energy reserves and reproductive function. It thus paved the way for the identification of factors involved in such an integrative control and of their potential pathophysiological implications. These later developments are illustrated by recent milestones in the area of reproductive biology, such as the discovery in the mid-1990s of the adipose hormone leptin and the clinical consequences, in terms of puberty onset and fertility, of human mutations in the genes encoding this ligand or its receptor.[23,24] More recently, a compelling candidate for a major central target or sensor of key metabolic signals governing the HPG axis has emerged in the form of the hypothalamic peptides, kisspeptins,[25] as a result of the discovery that loss-of-function mutations of their receptor (*KISS1*-derived peptide receptor, KISS1R, aka GPR54) were associated with hypogonadotropic hypogonadism and delayed or absent puberty in humans.[26,27]

In this chapter, we will review the main mechanisms whereby body energy stores and the metabolic state of the organism participate in the dynamic control of reproductive maturation and function. Because an extensive description of the interactions of metabolism and reproduction is available in the third edition of this book (at http://www.sciencedirect.com/science/article/pii/B978012515400050052X), this chapter will focus on recent work examining the biological effects on the reproductive axis of three key peripheral hormones and their central regulatory sensors, targeting directly or indirectly GnRH neuronal function. However, it is important to note that signals other than peripheral hormones are also involved in conveying metabolic information to the reproductive axis. For instance, direct nutrient sensing by specific cellular populations of the brain is known to also play an important role in the metabolic control of reproduction,[25] and ependymocytes lining the wall of cerebral ventricles in the hindbrain have been proposed as glucose sensors regulating the activity of GnRH neurons in the rat.[28] Likewise, catecholaminergic neurons located in the lower brainstem that project to the paraventricular nucleus (PVN) have been shown to sense and mediate the inhibition of the HPG axis induced by conditions of lipid deprivation.[29]

In this chapter, major attention will be paid to the mechanisms for the metabolic control of reproduction in females, yet comments on the metabolic regulation of male reproduction will be included where appropriate. Mention will also be made at the end of the chapter of the reproductive effects of some metabolic factors outside of the brain. In line with the theme of this book, emphasis will be placed on the review of physiological mechanisms, although some brief comments on pathophysiological implications will be also included when considered relevant. For further details on the adverse consequences of bidirectional metabolic–reproductive interactions, see Chapter 29.

METABOLIC HORMONES REGULATE REPRODUCTION: ROLES OF LEPTIN, INSULIN, AND GHRELIN

Since the formulation of the Frisch hypothesis, much effort has been devoted to deepen our understanding of the mechanisms responsible for the metabolic control of puberty onset and gonadotropin secretion in the adult, which has increased considerably in recent years. This has been mainly due to the discovery of several

neuroendocrine pathways whereby different peripheral hormones inform the reproductive centers of the brain about key parameters of the metabolic status of the body, such as the amount of energy (fat) reserves. In fact, it is well established that numerous hormones play important roles in transmitting metabolic information to the reproductive centers in both physiological and pathological conditions. For the sake of concision, I will mostly focus in this section on the physiological actions of the *classical* metabolic hormones, leptin, ghrelin, and insulin, in the control of the HPG axis; a synoptic overview of these regulatory effects is provided in Figure 35.1.

Leptin

Among the numerous endocrine regulators involved in the integrative control of reproduction and metabolism, leptin, a hormone identified in 1994 as a major secretory product of the white adipose tissue,[23] is universally recognized as an essential neuroendocrine integrator linking the magnitude of body fat stores with several neuroendocrine axes, including that regulating the reproductive system.[6,7,12,30] Leptin is secreted by the white adipose tissue in proportion to the size of body fat stores and acts as an anorexigenic and thermogenic factor at the hypothalamic level, thus contributing to dynamically adjusting energy requirements, fat reserves, and food intake.[12,30] Regarding reproductive control, numerous studies have settled the concept that leptin plays a central role in the metabolic control of puberty and fertility.[6,7,12,30,31] Accordingly, conditions of leptin insufficiency are often coupled to a delay or absence of puberty onset and perturbed fertility,[32] as observed in various animal models (e.g., the leptin-deficient ob/ob mouse, the leptin receptor–deficient db/db mouse, and the genetically obese Zucker fa/fa rat) as well as in human pathologies associated with low or null leptin levels.

Although an important role of leptin in the control of puberty was established soon after its identification in the mid-1990s, the precise nature of leptin effects on pubertal timing and subsequent fertility was initially the subject of considerable debate. Thus, early pharmacological studies in rodents suggested that leptin administration might actually induce or advance the onset of female puberty, therefore implying a net stimulatory action.[33,34] This possibility was relevant not only from a physiologic standpoint but also from a therapeutic perspective. Since leptin was considered for treatment of forms of morbid obesity of early origin, especially

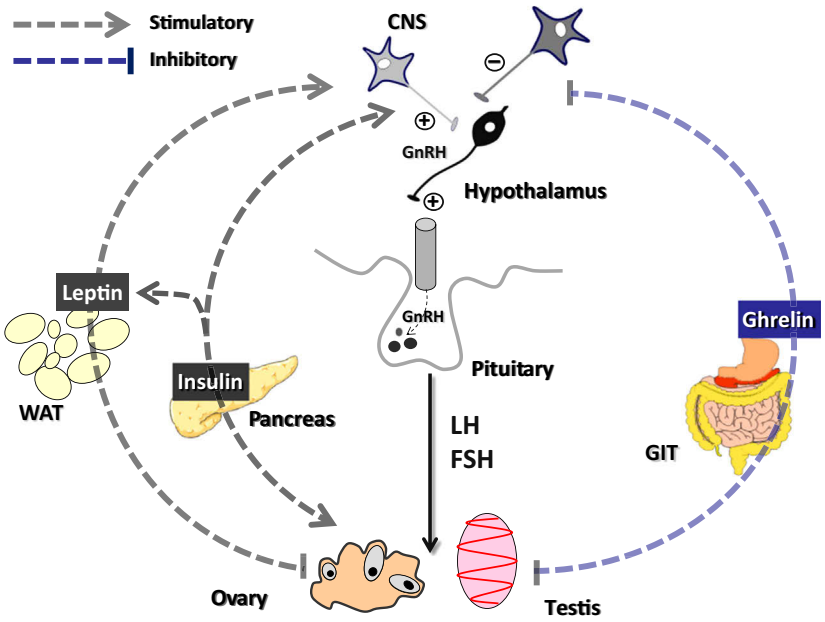

FIGURE 35.1 Schematic presentation of the regulatory roles of key hormonal signals, originating from different metabolic tissues, involved in the integrative control of food intake, energy balance, and reproductive function, including puberty. Paradigmatic examples of hormones with proven roles in the integrative control of food intake and the reproductive axis are presented, including factors from the adipose tissue (leptin: an inhibitory signal for food intake and a stimulatory or permissive signal for puberty and reproduction), pancreas (insulin; the same profile as leptin in terms of actions on food intake and reproduction), and gastrointestinal tract (ghrelin: a stimulatory signal for food intake and an inhibitory signal for the reproductive axis). These peripheral signals do not primarily target GnRH neurons, but rather operate via neuronal afferents that are either stimulatory (light gray) or inhibitory (dark gray). In addition to actions at central levels, inhibitory and stimulatory actions of these metabolic hormones directly at the pituitary (not depicted) and the gonads have been described. CNS: central nervous system; WAT: white adipose tissue; GIT: gastrointestinal tract.

in leptin-null individuals, any potential action of leptin to trigger precocious puberty could be considered an important adverse side effect and factor limiting the use of leptin in the management of childhood obesity. However, detailed studies of leptin administration in humans and rodents with leptin deficiency clearly documented that, while leptin is indispensable for puberty to proceed, leptin alone cannot trigger early puberty, therefore supporting a major permissive role for this hormone in the metabolic gating of pubertal maturation.[24,31,32,35–37] This implies that threshold leptin levels are mandatory, but not sufficient per se, to attain normal pubertal development and to maintain reproductive function in adulthood. Admittedly, this permissive function has been better characterized in females, whereas the role of leptin in male puberty and reproductive function remains less clear. In fact, circulating leptin has been shown to rise during the pubertal transition in girls,[38] while puberty onset in boys and male monkeys does not seem to correlate with a similar increase in leptin levels.[38,39] Moreover, high leptin levels, such as those seen in morbidly obese men, are frequently associated with hypogonadism,[40] yet the mechanism for such a paradoxical suppression of gonadal function in conditions of severe hyperleptinemia may involve central leptin resistance as well as direct inhibitory effects at the testicular level[40]; the latter will be discussed in more detail in the section Reproductive Effects of Metabolic Hormones Outside the Brain: Pituitary and Gonadal Actions of this chapter. In any event, conditions of leptin deficiency, such as those seen in ob/ob mice, are associated with detectable reproductive deficits in males,[4] whereas supraphysiological leptin replacement has been shown to increase testosterone and (modestly) gonadotropin secretion during short-term fasting in men,[41] thus suggesting that threshold leptin levels may be also needed to maintain proper reproductive function in males.

In spite of the compelling experimental and clinical data linking leptin and reproduction, recent findings suggest that particular aspects of the metabolic control of the gonadotropic axis may not involve a major role of leptin. For instance, apparent normalization of mean leptin concentrations in conditions of negative energy balance, such as short-term fasting, did not restore gonadotropin levels in female rats[42]; similar observations also have been reported in sheep, in which restoration of LH levels following exit of food restriction conditions took place in the absence of detectable changes of circulating leptin.[43] While leptin replacement in the above rat studies might not have reached optimal physiological levels, these observations represent a call of caution that warrants further analysis. In the same line, a recent preliminary report indicated that reducing glucocorticoid excess, which in general drives an inhibitory signal to the HPG axis, by means of genetic elimination of its hypothalamic stimulus, the corticotropin-releasing hormone (CRH), substantially ameliorates the reproductive phenotype of the ob/ob mouse, despite its persistent leptin deficiency.[44] Finally, it must be emphasized that the role and physiological relevance of leptin in the regulation of puberty, a period when full activation of the HPG axis takes place, versus fertility, in which leptin would operate as a signal for the maintenance of the capacity to reproduce during adulthood, might be different, so that the findings in one period may not be directly applicable to the other.

Insulin

Another peripheral hormone with a major role in the regulation of reproduction is the pancreatic hormone, insulin.[8] The key function of insulin as an essential metabolic regulator has long been universally recognized, and detailed recapitulation of its metabolic features clearly exceeds the aims and scope of this chapter. Notwithstanding, it is important to note for the purpose of this review that insulin operates as a major regulator of leptin synthesis, and, thus, their levels (and biological actions) are often tightly linked. In line with this contention, insulin has been proposed to also play a role as modulator of the HPG axis with predominant stimulatory actions, as clearly illustrated by the fact that conditions of low or null insulin levels, such as uncontrolled diabetes, are commonly associated with suppressed gonadotropin levels and various forms of reproductive failure.[8] Yet the reproductive *dimension* of insulin action remains less well characterized than that of leptin, despite the available evidence suggesting a relevant physiological role for insulin signaling in the regulation of the HPG axis.

In good agreement with the stimulatory/permissive role of insulin in the control of reproduction, a mouse line engineered to lack insulin receptors selectively in neurons has been shown to suffer not only hyperphagic obesity but also hypogonadism due to GnRH deficiency.[45] These features are remarkably similar to those seen in mouse models of leptin or leptin receptor deficiency. In addition, high peripheral insulin levels, achieved by hyperinsulinemic clamps, were able to significantly increase LH secretion, regardless of the actual glucose levels, therefore suggesting a primary role of changes in insulin concentrations in this phenomenon.[46] In line with these rodent studies, recent analyses during the menstrual cycle in healthy women have demonstrated that a rise of insulin concentrations peripherally can evoke a significant increase in the frequency of LH pulses.[47] Altogether, these findings support a stimulatory role of insulin in the control of the gonadotropic axis. As a call of caution, however, it remains possible that some of the positive effects of insulin on the reproductive system may stem from its ability to stimulate leptin secretion. In

addition, the physiological roles of insulin in the control of key reproductive events, such as puberty onset or the preovulatory surge of gonadotropin, remain ill-defined and warrant further experimentation.

Ghrelin

During the last decade, compelling evidence has been presented supporting an essential role of ghrelin in the control of food intake and energy balance, ghrelin being mainly produced in the stomach to act as the (only known circulating) orexigenic factor.[48] The metabolic dimension of ghrelin is reinforced by the demonstration of its proven role in glucose homeostasis and insulin secretion,[49,50] its central effects on energy expenditure and nutrient partitioning, and its interactions with key signals in energy homeostasis, such as leptin.[51] To further reinforce the metabolic relevance of ghrelin, its circulating levels are in most cases negatively correlated with the body mass index,[49] and hence ghrelin has been proposed to operate as a long-term signal for energy insufficiency, with reciprocal interactions (in a yin-and-yang manner) with leptin, as a signal of energy abundance.[51] In good agreement, ghrelin has been documented to play a regulatory role in the control of the HPG axis, with a predominant inhibitory action that is opposite to the permissive and stimulatory effects of leptin.

In line with its function as a signal of energy insufficiency, experimental studies conducted mainly in rodents have demonstrated a modulatory role of ghrelin in puberty onset, with predominant inhibitory actions; thus, acute elevation of ghrelin concentrations suppressed circulating gonadotropin levels, and chronically elevated ghrelin levels delayed different indices of pubertal maturation.[22,52–54] Interestingly, studies of the rat suggested that females are less sensitive than males to the inhibitory effects of ghrelin on pubertal maturation.[55] In any event, administration of high doses of ghrelin to female rats during puberty was able to delay this process, based on external (i.e., vaginal opening) and ovarian signs of puberty.[32] The fact that male rodents appear to be more sensitive than females to the inhibitory actions of ghrelin on puberty is opposite to the reported effects of leptin, as females seem to be more sensitive to the regulatory actions of leptin on puberty onset.[6] Whether, as described for the rat, human puberty is sensitive to the effects of ghrelin is yet to be defined. In this regard, a progressive decline in the circulating levels of ghrelin during puberty in humans has been demonstrated.[56] Hence, it is tempting to hypothesize that if ghrelin operates as an inhibitory signal for the onset of puberty in humans, such a decrease in its plasma levels may play a permissive role in pubertal onset.

Compelling experimental evidence indicates that ghrelin is also an inhibitory signal for the gonadotropic system in adulthood. In brief, studies conducted in several species have documented that, after its systemic or intracerebral administration, ghrelin can induce significant inhibition of LH, and to a much lesser extent FSH, secretion. While these studies were initially conducted in rodents, the ability of ghrelin to suppress the gonadotropic axis has since been confirmed in sheep, nonhuman primates (ovariectomized rhesus monkeys), and humans.[53,57–64] In rats, the inhibitory actions of ghrelin on LH secretion have been observed in males and females, after both acute and repeated injection.[53–55,57,58] In good agreement, central administration of ghrelin has been shown to induce a significant reduction of ovarian steroids, estrogen and progesterone, in cyclic female rats,[65] while chronic administration of ghrelin to adult male rats during undernutrition (hence already displaying an elevation of endogenous ghrelin levels) evoked a marked drop in serum LH and testosterone levels.[66] Likewise, in the intact sheep and gonadectomized monkey, ghrelin has been shown to suppress different aspects of the pulsatile secretion of LH.[59,60] Finally, ghrelin infusions have been reported to partially inhibit spontaneous LH pulsatility in healthy men,[61,62] and negative correlations between ghrelin concentrations and LH, FSH, testosterone, and estradiol levels have been detected in young men suffering beta thalassemia, a condition linked to perturbations of puberty and gonadotropic function.[67] On the other hand, another study did not detect significant changes in circulating LH levels following an injection of a single bolus of ghrelin in healthy cyclic women[68]; this might suggest a sexual dimorphism in the sensitivity to the suppressing effects of ghrelin on the gonadotropic axis, with adult males being more sensitive than females, in line with data from pubertal studies.

Other Peripheral Hormones

Despite the prominent roles of leptin, insulin, and ghrelin in transmitting metabolic information to the HPG axis, as described in this chapter, the available evidence strongly suggests that other peripheral hormones, with important roles in the control of metabolism, may also participate in the regulation of various facets of reproductive maturation and function. However, the physiological significance of these additional regulatory axes (whose existence, in some cases, was not documented until very recently) remains to be assessed. As a paradigmatic example, the bone product, osteocalcin, which is known to play an important role in metabolic homeostasis by virtue of its ability to regulate insulin sensitivity and circulating glucose levels,[69,70] recently has been shown to directly regulate testicular function,[71] therefore suggesting the existence of a previously unsuspected hormonal bone–gonadal axis that may contribute to link the metabolic status and the function of the HPG axis. In

the same vein, adipokines other than leptin have been implicated in the control of the HPG axis. Although, for the sake of concision, the experimental data supporting a role of those adipose-born cytokines in the regulation of the reproductive system are not thoroughly reviewed in this chapter, it is worth mentioning that adiponectin, whose levels fluctuate in a reciprocal manner with leptin, has been shown to suppress GnRH secretion by GT1–7 cells[72,73] and to inhibit basal LH secretion and GnRH-induced LH release at the pituitary–gonadotrope level.[74,75] Similarly, other gut-derived hormones may cooperate with ghrelin in the central control of the HPG axis. Thus, glucagon-like peptide 1 (GLP-1), whose roles as an incretin factor and major regulator of postprandial insulin secretion and glucose homeostasis are unanimously recognized,[76] has been shown to stimulate GnRH secretion by GT1–7 cells and to modestly increase LH levels after central administration.[77] Yet, the physiological and pathophysiological relevance of those regulatory functions on the elements of the HPG axis awaits further investigation. Likewise, the role of circulating growth factors, such as insulin-like growth factor I (IGF-I), in the control of the reproductive axis, and particularly of GnRH neurons, has been well documented (for further details, see the section Metabolic Targeting of GnRH Neurons: Direct Actions and Indirect Pathways).

In the same vein, gonadal hormones, such as 17β-estradiol, which play an essential role in the control of GnRH neuronal function, are also known to serve important roles in the control of metabolism and energy homeostasis.[78] Thus, ovariectomy causes an important increase in body weight in laboratory rodents, and loss of estrogens also may underlie the body weight alterations (mainly weight gain) frequently observed after menopause.[78] While such metabolic effects of estrogen have been long recognized, the neuroendocrine basis for these actions remains poorly characterized. Yet, recent studies have helped to dissect some of the central components for the joint metabolic and reproductive effects of estrogens, which seem to operate in the arcuate nucleus (ARC) to suppress feeding and conduct negative feedback, while actions in the ventromedial hypothalamus (VMH) appear to mediate at least part of the effects of estrogen on thermogenesis and ovulation.[79] Thus, elimination of estrogen receptor alpha (ERα) signaling in steroidogenic factor 1 (SF1) neurons in the VMH has been shown to induce obesity, hypometabolism, and anovulation, while ablation of ERα in proopiomelanocortin (POMC) neurons in the arcuate caused hyperphagia, perturbation of negative feedback of gonadotropins, and infertility.[79] Further details on the nature of the neuronal populations mediating the reproductive and metabolic effects of estrogens are provided in the sections Other Products of KISS1 Neurons in the Metabolic Control of Reproduction: Role of KNDy Neuropeptides and Other Central Transmitters in the Metabolic Control of Reproduction of this chapter.

METABOLIC TARGETING OF GnRH NEURONS: DIRECT ACTIONS AND INDIRECT PATHWAYS

As reviewed in the previous section, peripheral hormones modulate the maturation and function of the HPG axis. While compelling experimental data have documented the ability of these signals to act at levels of the reproductive axis other than the brain (for further details, see the section Reproductive Effects of Metabolic Hormones Outside the Brain: Pituitary and Gonadal Actions), it is now recognized that a substantial component of their reproductive effects occurs at central levels, specifically via modulation of GnRH neuronal functions, as the major output pathway for the upstream regulatory elements of the gonadal axis. This has been clearly illustrated by functional genomic analyses showing that neuronal elimination of the receptors for insulin or leptin impairs puberty onset and fertility,[45,80] and by data documenting the ability of leptin, insulin, and ghrelin to modulate GnRH secretion by hypothalamic explants ex vivo or primary cell cultures in vitro.[7,8,81] There has been, however, a significant debate on whether the actions of such metabolic hormones are exerted directly on GnRH neurons or, rather, primarily operate through intermediary neuronal pathways. Such a precedent for an indirect control of GnRH neuronal activity has been long recognized. Thus, despite their central role in the control of the gonadotropic system, it has been known for years that GnRH neurons are devoid of receptors for some of their major regulators, such as ERα, which is responsible for the feedback effects of estradiol,[82] therefore suggesting that such an *indirect* sensing is commonplace for this neuronal population.[83]

In fact, elegant studies using Cre–LoxP approaches support the contention of a predominantly indirect mode of action of leptin and insulin in the control of GnRH function. Thus, studies from Anderson and colleagues showed that while neuron-specific knockout (KO) of leptin receptors prevented normal puberty onset and caused infertility in male and female mice, selective elimination of leptin receptor expression from GnRH neurons was compatible with normal puberty onset in females and preserved fertility.[80] This phenomenon is in good agreement with the observation that intracerebral injection of leptin caused robust expression of signal transducer and activator of transcription 3 (STAT3) in specific hypothalamic areas linked to the generation of the preovulatory surge of gonadotropins, such as the anteroventral periventricular (AVPV) nucleus, but not in GnRH neurons.[80] Moreover, GnRH neurons appear to

be devoid of functional leptin receptors in physiological conditions, according to brain expression studies in rodents and primates.[84,85]

Similarly, Wolfe and coworkers recently demonstrated that selective ablation of insulin receptors from GnRH neurons failed to induce any detectable alteration in the timing of puberty or adult reproductive function in male and female mice.[86] Again, this is in contrast to the overt reproductive phenotype of neuron-specific insulin receptor-null mice, which displayed central hypogonadotropic hypogonadism.[45] Although results from congenital KO should be interpreted with caution because of the possibility of developmental compensatory mechanisms, these findings suggest that direct leptin or insulin signaling in GnRH neurons is dispensable for the development and maintenance of normal reproductive function, at least in rodents. These results thus point out the existence of indirect pathways, sensitive to these key metabolic hormones, which would operate as conduits for the transmission of such metabolic information to GnRH neurons.

Nonetheless, evidence for the direct effects of key metabolic hormones, such as leptin and insulin, on GnRH neurons has been obtained from studies using primary hypothalamic cultures and different GnRH neuronal cell lines. Thus, analyses in the immortalized GnRH-secreting cell line, GT1–7, revealed that these cells express functional leptin receptors and respond to low doses of leptin with increases in GnRH release.[87] In the same vein, the clonal GnRH neuronal cell line, GnV3 (conditionally immortalized GnRH cells), was shown to express the insulin receptor and to respond to insulin stimulation with increases in *GnRH* mRNA expression,[88] a phenomenon that also has been documented in GnRH-expressing GnRH-secreting neuronal 11 (GN11) cells.[89] Furthermore, detailed molecular studies have documented the involvement in this phenomenon of signaling cascades known to mediate insulin effects in peripheral metabolic tissues, such as the mitogen-activated protein kinase extracellular-regulated kinase (Erk) 1/2.[87] It is emphasized, however, that the relevance of these observations is hampered by the fact that the immortalized cell lines used in the above studies might not be representative of truly physiological conditions. In fact, data using functional genomics have challenged the concept of direct GnRH effects of leptin and insulin (as discussed here). The possibility of compensatory mechanisms in models of congenital KO of specific receptors, however, does not allow one to completely rule out that some direct effects of these signals in GnRH neurons might occur under certain conditions. On the other hand, GnRH neurons seem to be sensitive to the direct regulatory actions of other metabolic and growth factors involved in the control of puberty, such as IGF-I, since selective elimination of IGF-I receptors from GnRH neurons resulted in delayed puberty onset in male and female mice.[86]

In contrast to leptin and insulin, the mechanisms whereby ghrelin participates in the central control of the gonadotropic axis remain only superficially studied. Pharmacological analyses have suggested that the inhibitory effects of ghrelin on the this axis occur, at least partially, at the hypothalamic level, since ghrelin has been shown to inhibit GnRH secretion by hypothalamic fragments from female rats challenged with the hormone ex vivo,[53] and systemic administration of ghrelin caused a reduction in GnRH pulse frequency by hypothalamic explants from immature and young adult male rats in vitro.[90] Whether this central action of ghrelin is conducted directly on GnRH neurons or via indirect pathways is yet to be fully defined. Circumstantial evidence suggests an indirect mode of action of ghrelin, since its inhibitory effects on LH secretion in the monkey were inhibited by a nonselective antagonist of CRH,[91] which is a suppressor of the HPG axis. In addition, ghrelin was able to inhibit at least part of the effects of potent stimulators of gonadotropin secretion acting at central levels, such as naloxone in humans and kisspeptin in rats.[54,62] The latter, however, is compatible with a direct action of ghrelin on GnRH neurons to suppress their responsiveness to kisspeptins. For further details on the roles of kisspeptins and the neurons that synthesize these peptides in the metabolic control of the reproductive axis, see the section KISS1 Neurons as Central Relays of Metabolic Information: Expression and Functional Studies.

KISS1 NEURONS AS CENTRAL RELAYS OF METABOLIC INFORMATION: EXPRESSION AND FUNCTIONAL STUDIES

The recognition of the existence of indirect pathways responsible for transmitting metabolic information to GnRH neurons set the scene for the identification of the afferent neuronal circuits responsible for such a function. In this context, considerable efforts have been devoted in recent years to elucidate the putative roles of kisspeptins, the products of the *Kiss1* gene, in the metabolic control of puberty and fertility.[1,2] Kisspeptins are a family of structurally related peptides of the Arg–Phe (RF)-amide superfamily that act via the surface receptor, KISS1R (aka GPR54).[1] Different molecular forms of kisspeptins, varying in amino acid length, have been reported, which include kisspeptin 54 and kisspeptin 10; the latter is the shortest peptide fragment with the capacity to fully activate KISS1R. In terms of nomenclature, we will adhere to recent proposals,[1,92] whereby *Kiss1* and kisspeptins will be used to name the gene–mRNA and proteins, respectively, while the term "KISS1 system" will be employed to globally refer to the ligand-receptor system.

As summarized in Chapter 11 on GnRH neuronal network, during the last few years, neurons producing kisspeptins (referred hereafter as "KISS1 neurons") in the basal forebrain have been recognized as master elements of the reproductive brain, which operate as essential conduits for transmitting the regulatory actions of numerous modulators of the HPG axis, from gonadal steroids to photoperiodic cues.[1,83,93] As reviewed in this section, a number of expression and functional studies conducted in different (mainly rodent) species during recent years have documented that the hypothalamic KISS1 system is sensitive to different forms of metabolic stress known to perturb puberty and gonadotropin secretion.[2,94] These observations, together with the fact that exogenous administration of kisspeptin, as a means to replace its defective endogenous levels, can ameliorate the reproductive deficits seen in those conditions, support a major role of kisspeptin signaling in the metabolic control of reproduction.[2,94] However, recent evidence suggests also that the participation of KISS1 pathways in this phenomenon is probably not as straightforward as initially anticipated, and apparently discrepant findings on the contribution of KISS1 circuits in mediating the effects of key metabolic hormones, such as leptin, have been reported in the last few years. The latter will be critically discussed in the section Leptin Signaling and KISS1 Neurons: Direct, Indirect, or Independent Effects?

Expression Studies

Studies conducted initially in this area were mainly focused on the analysis of changes in *Kiss1* expression in models of negative energy balance, which are linked to different degrees of reproductive suppression. Thus, pubertal male and females rats under short-term fasting were shown to display a significant decrease in the hypothalamic expression of *Kiss1* mRNA, which was coupled to a significant drop of serum LH levels.[95] Similar observations were later reported in adult female rats[96,97] and adult male mice.[98] Suppression of the hypothalamic expression of *Kiss1* has also been described in other models of metabolic stress coupled to negative energy balance and impaired reproductive function, such as uncontrolled experimental diabetes; for example, male and female rats injected with streptozotocin suffered central hypogonadism associated with a marked reduction in hypothalamic *Kiss1* mRNA expression levels.[99,100]

Although these expression studies documented the sensitivity of the hypothalamic KISS1 system to changes in body energy stores and metabolic cues, several aspects of this regulation remained initially uncharacterized. For instance, it is well known that there are two distinct kisspeptin neuronal populations, located in the ARC and the AVPV, which in rodents respond differentially to key reproductive modulators, such as sex steroids; for further details, see Refs 1,83 and Chapter 11 on the GnRH neuronal network and Chapter 26 on control of the ovarian cycle of the rat and mouse. However, the relative sensitivity of the two populations of KISS1 neurons to metabolic regulation is yet to be fully elucidated. In this context, female rats submitted to persistent underfeeding during the pubertal transition display a significant suppression of *Kiss1* mRNA expression in the ARC.[101] Similarly, leptin-deficient ob/ob male mice also demonstrated a decrease in *Kiss1* mRNA levels in the ARC.[102] On the other hand, adult OVX female rats replaced with estrogen and submitted to short-term fasting had reduced *Kiss1* mRNA levels at the AVPV,[103] whereas 50% caloric restriction of adult female rats resulted in a drop of *Kiss1* mRNA levels in both the ARC and the AVPV.[42] An analogous decrease in *Kiss1* mRNA levels in the AVPV has been reported in rats during lactation,[104] another condition of negative energy balance. Finally, dietary restriction in sheep induced a profound inhibition of *Kiss1* mRNA expression in the ARC and preoptic area (POA)[105]; the latter is rostral to the hypothalamus and, as the AVPV, has been reported to hold KISS1 neurons that might be involved in positive feedback and the control of ovulation.[106] These observations suggest that KISS1 neurons in both the ARC and AVPV (or equivalent rostral areas in other species) are subjected to metabolic regulation, although their relative sensitivity to conditions of metabolic stress may differ from each other.

It is also interesting to note that, while most of the analysis on the influence of metabolic signals on the KISS1 system has focused on changes in hypothalamic mRNA levels, whether such changes translate into alterations of kisspeptin content or, eventually, kisspeptin release remains ill defined. As illustrated in Figure 35.2(A), immunohistochemical (IHC) studies in pubertal female rats have described that short-term fasting induces a significant decrease of kisspeptin immunoreactivity (IR) and the number of kisspeptin-positive neurons in the ARC.[2] In the same vein, prolonged conditions of negative energy balance, such as lactation, also have been shown to induce a drop in kisspeptin IR in the ARC.[104] Indeed, the ARC population of KISS1 neurons is also sensitive to other forms of stress, as documented by the decrease in the number of kisspeptin IR cells detected in adult male rats submitted to an acute inflammatory challenge by administration of bacterial lipopolysaccharide (LPS).[107] In any event, some studies have failed to detect an overt decrease in kisspeptin IR in adult female rats after a 48-h period of food deprivation,[2] although this study was conducted in gonadal-intact animals, and compensatory changes in kisspeptin expression might have occurred following fasting-induced changes in the endogenous sex steroid milieu.[2] Furthermore, the lack of direct assessment of kisspeptin release may render IHC

data of limited value, since low kisspeptin IR may be compatible with either enhanced kisspeptin release (and hence signaling) or low synthesis and reduced release (and hence signaling).[104]

Of important note, most of the expression analyses addressing the metabolic regulation of the hypothalamic KISS1 system have been conducted in models of negative energy balance (see this chapter), in order to examine the principle that KISS1 neurons are sensitive to metabolic signals. In contrast, a very limited number of studies have produced data on the impact of situations of persistent overweight and obesity on the hypothalamic expression of *Kiss1*. These analyses, however, are of considerable interest because of the escalating prevalence of obesity and its potential reproductive comorbidities, which include perturbed pubertal timing and subfertility.[18–21] In this context, initial studies in long-term diet-induced obese male mice of the C57 black 6 strain documented that these animals had largely preserved hypothalamic *Kiss1* mRNA expression, despite very low levels of circulating testosterone.[98] Although *Kiss1* mRNA levels in this study were assessed in whole hypothalamic fragments, *Kiss1* expression was likely located in the ARC because male rodents have a prominent ARC KISS1 population but rather few KISS1 neurons in the AVPV.[83] While this finding might suggest a lack of impact of obesity on the hypothalamic KISS1 system, it must be considered that the severely decreased testosterone levels observed in these diet-induced obese mice should have evoked an increase in the hypothalamic expression of *Kiss1* mRNA,[83] which actually did not take place. Hence, these observations strongly suggested that obesity is associated with the impairment of KISS1 neurons to respond appropriately to major regulators, such as sex steroids.[98]

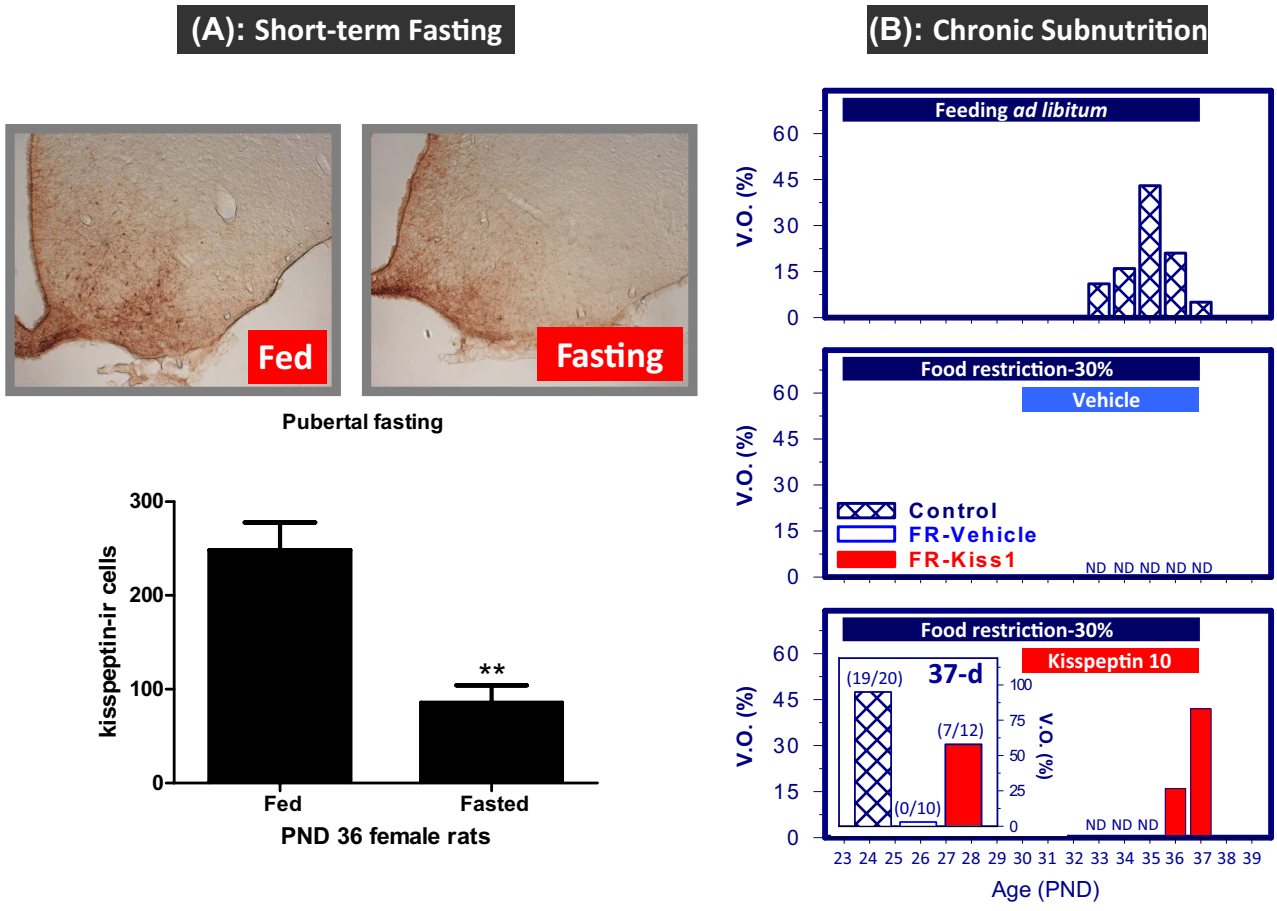

FIGURE 35.2 **Impact of conditions of metabolic stress on the hypothalamic KISS1 system and its functional consequences in pubertal female rats.** In the *left panels* (A), the suppressive effect of short-term fasting on the number of kisspeptin-immunoreactive neurons in the ARC is shown. In the *right panels* (B), the consequences of kisspeptin replacement in terms of pubertal onset in a model of chronic subnutrition (FR: a 30% reduction in daily food ration) during puberty is illustrated. This protocol of partial food deprivation caused a 30% decrease in body weight (BW) gain and prevented vaginal opening (VO), an external sign of puberty. However, repeated intracerebral injections of kisspeptin 10, in the face of a persistent reduction in BW, was sufficient to rescue VO in >60% of the animals; see the accumulated percentage of VO in the experimental groups on PND37 in the inset of the lower graph of Panel (B). In addition, potent gonadotropic and estrogenic responses were evoked by kisspeptin 10 in all treated females (data not shown). PND: postnatal day (age); ND: not detectable; d: day. *Source: Taken from Refs 4,81, with modifications.*

In the same vein, a more recent study has shown that persistent obesity in DBA/2J mice, a mouse strain prone to obesity-induced infertility, caused a marked reduction of *Kiss1* mRNA levels in the ARC and AVPV, as well as in the number of KISS1 neurons in the AVPV.[108] These observations would suggest that conditions of persistent overweight can suppress the hypothalamic KISS1 system, as we have also very recently observed in adult male rats subjected to various obesogenic insults (Sanchez-Garrido & Tena-Sempere, submitted).

Of note, the impact of obesity on the hypothalamic KISS1 system is likely complex and does not always involve clear-cut inhibitory effects. For instance, male rats transiently exposed to a high-fat diet displayed an elevation of hypothalamic *Kiss1* mRNA levels.[96] Similarly, immature female rats fed on a high-fat diet during the pubertal transition displayed an advancement in the age of vaginal opening coupled to an increase in *Kiss1* mRNA expression in the ARC and medial POA.[109] Moreover, recent studies addressing the effects of early postnatal overfeeding in female rats described a potential correlation between early-onset overweight, advanced pubertal maturation, and increased *Kiss1* mRNA levels in the hypothalamus, as well as possibly increased numbers of kisspeptin fibers in the AVPV.[110] Admittedly, however, the latter observations regarding *Kiss1* mRNA levels or kisspeptin projections have not been fully replicated by other studies.[111,112] Altogether, the above data from different models of overweight/obesity in rodents strongly suggest that, depending on the age, duration, and/or degree of obesity, stimulatory or inhibitory responses in terms of *Kiss1* expression and gonadotropic function can take place. However, the mechanisms for such a switch in the responses of the KISS1 system, and to what extent these are similar to or distinct from those affecting the HPG axis in conditions of energy insufficiency, remain to be defined.

Functional Analyses

The capacity of extreme metabolic conditions to affect the expression of *Kiss1*/kisspeptin in discrete hypothalamic areas would anticipate that functional changes in the endogenous kisspeptin tone occur in those conditions, which would be responsible, at least in part, for the associated reproductive arrest. However, direct assessment of actual changes in kisspeptin release in situations of metabolic stress has not been conducted. Instead, proof-of-principle studies have been used to evaluate whether pharmacological administration of kisspeptin, as a means to prevent the expected reduction of endogenous levels in situations of energy insufficiency, could rescue different reproductive parameters in those situations. This might provide indirect evidence for a role of kisspeptin signaling in the metabolic control of the HPG axis. Yet, it must be noted that such proof-of-principle analyses have been mostly conducted in rather extreme metabolic conditions and used protocols of central administration of high doses of kisspeptin, which might have caused an elevation of the endogenous kisspeptin tone over the expected physiological levels. Hence, results must be interpreted with caution.

Despite these potential reservations, various pharmacological studies have shown that by enhancing the endogenous kisspeptin drive in conditions of energy insufficiency and metabolic stress, without any other neuroendocrine manipulation, several indices of pubertal and reproductive failure can be rescued. For instance, as shown in Figure 35.2(B), repeated intracerebral injections of kisspeptin 10 to immature female rats, submitted to a protocol of chronic subnutrition after weaning that caused pubertal arrest, was sufficient to induce vaginal opening in a significant proportion of cases, together with robust gonadotropic and estrogenic responses (not shown in Figure 35.2(B)), that overrode the prevailing suppression of circulating levels of gonadotropins due to malnutrition.[95] Similarly, repeated administration of kisspeptin 10 to hypogonadotropic diabetic male rats ameliorated testicular and prostate weights, and normalized circulating LH and testosterone levels, while central injection of kisspeptin 10 to uncontrolled diabetic female rats was able to reverse their hypogonadotropic state.[99,100] In the same vein, a recent study has documented that 11-h infusions of kisspeptin 10 are able to potently stimulate LH and testosterone secretion in men with hypogonadism associated with type 2 diabetes.[113] Of note, in the studies in models of metabolic stress discussed here, the rescue of reproductive indices was achieved in spite of the apparent lack of direct effects of kisspeptin on metabolic parameters, such as body weight or circulating glucose levels—observations that reinforce the primary role of kisspeptin signaling as the central effector for the metabolic regulation of the reproductive axis.

Interestingly, assessment of acute gonadotropin responses to kisspeptin administration has revealed additional changes in the functionality of the KISS1 system in different conditions of metabolic stress. For instance, LH secretion following bolus injection of kisspeptin 10 to fasted male and female rats was not only preserved but even enhanced, despite the lowering of basal gonadotropins levels due to food deprivation.[95,114] Furthermore, a higher responsiveness to kisspeptin 10 in vitro, in terms of GnRH secretion, has been detected in hypothalamic explants from fasted rats,[95] and the duration of LH responses to the continuous infusion of kisspeptin 10 in vivo was prolonged in female rats subjected to chronic subnutrition.[115] All in all, these findings are suggestive of a state of hyperresponsiveness to kisspeptin in situations of negative energy balance.

A plausible mechanism for this phenomenon is that conditions of negative energy balance, which are associated with a putative decrease of the endogenous kisspeptin tone (see the section Expression Studies), would induce a compensatory state of augmented responsiveness via an increase in the expression of KISS1R. In fact, protracted fasting in pubertal rats evoked an increase in their hypothalamic *Kiss1r* mRNA levels.[95] However, it is emphasized that shorter periods of fasting in adult mice have been shown to reduce *Kiss1r* mRNA expression.[98] It should also be noted that some of the changes discussed here in responses to kisspeptin administration may derive from a greater accumulation of GnRH and/or gonadotropin stores due to continued synthesis of peptide–protein in an absence or reduction in release due to the prevailing state of negative energy balance. Overall, the available evidence strongly suggests that metabolic signals not only modulate *Kiss1*–kisspeptin expression in the hypothalamus but also can influence GnRH–gonadotropin responsiveness to the stimulatory effects of kisspeptins. The nature of such regulatory actions, however, may vary among species, since it has been reported that, contrary to rodents, fasting decreases testosterone responses to kisspeptin stimulation in the monkey.[116]

LEPTIN SIGNALING AND KISS1 NEURONS: DIRECT, INDIRECT, OR INDEPENDENT EFFECTS?

The demonstration of the impact of various conditions of metabolic stress on KISS1 neurons led to specific analyses aimed at the identification of the putative signals responsible for the metabolic control of this system. Because of leptin's paramount physiological relevance in the metabolic gating of puberty and fertility (see the section Leptin), considerable efforts in this area have been devoted to assessing the specific roles of this hormone in the control of the KISS1 system as a putative pathway to transmit its stimulatory and facilitatory effects on GnRH neuron activity.[6,7,12,117] Indeed, in the last few years, convincing evidence has been presented to document the capacity of leptin to modulate *Kiss1* expression. Yet, it is noted that most of the studies reported to date have involved the use of rather extreme models of leptin deficiency or, in some cases, immortalized cell lines. Moreover, the use of high doses of leptin, as a proof-of-principle approach, may limit the physiological relevance of some of the reported stimulatory effects of leptin on the KISS1 system under certain conditions, such as the exit from situations of negative energy balance.[42] While these features do not invalidate the majority of the experimental studies on this topic reported so far, they bring a call of caution when directly extrapolating some of the experimental observations to physiological conditions.

Studies in gonadectomized ob/ob mice were the first to establish that conditions of leptin deficiency induce a marked suppression of *Kiss1* mRNA levels in the ARC, while leptin administration to these animals enhanced *Kiss1* mRNA expression in this nucleus.[102] Similarly, central infusion of leptin to male rats with uncontrolled diabetes was able to normalize hypothalamic *Kiss1* gene expression, along with the restoration of LH and testosterone levels, in this model of hypogonadotropic hypogonadism associated with marked hypoleptinemia and hypoinsulinemia.[99] In line with these in vivo findings, leptin has been shown to increase *Kiss1* gene expression in the murine hypothalamic cell line N6,[98] as well as in primary cultures of human fetal GnRH-secreting neuroblasts.[118]

In line with the ability of leptin to modulate hypothalamic *Kiss1* expression, initial studies in ob/ob mice demonstrated the expression of the gene encoding leptin receptors in a significant proportion (>40%) of KISS1 neurons in the ARC.[102] Of note, however, a later study, using a transgenic mouse line that allows green fluorescent protein tagging and identification of KISS1 neurons in vivo, reported that just a modest fraction of KISS1 neurons express functional leptin receptors, as evidenced by the fact that only a subset of <15% of ARC KISS1 neurons (and none of the AVPV KISS1 neurons) appeared to display conventional phosphorylated STAT3 (pSTAT3) responses to leptin treatment,[119] pSTAT3 being the major transduction signal for leptin actions in the brain.[120] The later findings are consistent with a predominant indirect mode of action of leptin on KISS1 neurons, as proposed recently (discussed in this chapter).

Analogous findings, supporting a role of leptin in the control of the KISS1 system, have been reported in other species. For instance, studies in sheep have demonstrated the expression of the leptin receptor gene in both ARC and POA KISS1 neurons, and leptin has been shown to enhance *Kiss1* mRNA expression in those hypothalamic nuclei.[105] Likewise, a recent study reported that in the guinea pig, ARC KISS1 neurons, which express leptin receptors, are depolarized by leptin via activation of transient receptor potential channels.[121] As a whole, these observations, coming from different species and experimental models, do support the existence of a leptin–kisspeptin connection, whereby leptin actions could be signaled onto GnRH neurons via modulation of KISS1 afferents, thereby allowing proper maturation and function of the HPG axis in conditions of energy (and, hence, leptin) abundance.

Despite the solid evidence summarized here, the concept that leptin acts directly on KISS1 neurons has been challenged recently by two independent studies. First, using a Cre–LoxP strategy, the Elias team has published

that congenital elimination of leptin receptors selectively from *Kiss1*-expressing cells does not prevent pubertal maturation or attainment of fertility.[122] It must be noted, however, that congenital ablation of leptin receptors from KISS1 neurons might have induced developmental compensatory changes that could reduce or eliminate the phenotypic impact of such ablation. Second, a recent independent study, mapping the distribution of leptin receptors in different hypothalamic areas in the mouse and sheep, could not find evidence for their expression in KISS1 neurons except for a small population in the ARC.[123] It must be noted, however, that in this study the number of kisspeptin-positive cells in the ARC was surprisingly low, and therefore this population may have been underestimated and hence, also, the degree of co-expression of kisspeptin and leptin receptors.[123] Interestingly, the latter work identified a previously unknown population of neurons expressing leptin receptors, located in the ARC and AVPV, in the close vicinity of KISS1 neurons.[123] These newly identified leptin receptor-expressing neurons may participate in transmitting leptin effects to KISS1 neuronal populations. All in all, the combination of such expression and functional genomic studies strongly suggests that a substantial component of the effects of leptin on the hypothalamic expression of *Kiss1*/kisspeptin may be conducted indirectly and transmitted via intermediate pathways of as-yet-unknown nature.[123] The physiological significance of such an intricate, indirect mode of regulation of KISS1–GnRH neurons by leptin, and its importance relative to direct KISS1 regulatory effects of leptin in various species, including humans, is yet to be elucidated. It must be kept in mind, though, that species differences might exist regarding the mode of action of leptin on KISS1 neurons, as indirectly suggested by the apparent differences reported in the fraction of KISS1 neurons expressing leptin receptors in the mouse,[119] guinea pig,[121] and sheep.[105]

Notwithstanding the presumed important roles of KISS1 neurons in transmitting metabolic information in general, and leptin effects in particular, to GnRH neurons, there is solid experimental evidence that the reproductive actions of leptin are, in part, conveyed via primary actions at hypothalamic areas that do not apparently contain KISS1 neurons. This raises the possibility of the existence of kisspeptin-independent pathways for transmitting leptin-metabolic effects to the GnRH neuronal network.[5] A paradigmatic example in this sense is the ventral premammillary nucleus (PMV). In fact, this nucleus had been suggested as an important center for the transmission of different environmental cues to the reproductive axis years before the identification of the roles of leptin and kisspeptins were established.[4] Recently, the PMV has been pointed out as a primary target area for the effects of leptin on puberty and fertility.[122,124] Thus, a subset of neurons in the PMV has been shown to express leptin receptors and is excited following leptin stimulation. In addition, bilateral excitotoxic lesions of the PMV lowered LH and estradiol levels, and prevented the positive effects of leptin on the gonadotropic axis in fasted animals. It must be noted, however, that since only a fraction of PMV neurons expresses leptin receptors, it is possible that part of the reproductive phenotypes of these excitotoxic lesions may stem from the damage of leptin-independent pathways. In any event, selective re-expression of leptin receptors in the PMV of leptin receptor-deficient mice was able to induce puberty and improve fertility, specifically in female mice.[4,5] Of note, a similar procedure for leptin receptor rescue of the PMV in male mice was ineffective in terms of recovery of puberty onset,[122] an observation that suggests important sex differences in the role of PMV circuits in mediating the reproductive actions of leptin.

Taken together, the observations discussed here would suggest the existence of KISS1-independent circuits for the transmission of leptin effects onto GnRH neurons. Importantly, this evidence does not refute a critical role of kisspeptins in the metabolic control of reproduction, nor does it rule out the possibility of interactions between these alternative circuits and KISS1 pathways. In fact, neuroanatomical data suggest that neurons from the PMV innervate kisspeptin and GnRH neurons, and very recent data have documented that lesions of the PMV disrupt the expression patterns of *Kiss1* and *GnRH* mRNA during the preovulatory period in female rats[125]; therefore, it is highly plausible that leptin-sensitive pathways originating from the PMV may impinge onto KISS1 circuits for transmitting part of the reproductive actions of leptin. A schematic representation of the potential pathways whereby leptin transmits its regulatory effects onto GnRH neurons, via KISS1 by direct or indirect actions or via KISS1-independent actions, is provided in Figure 35.3.

Of note, the ability of leptin to modulate the hypothalamic KISS1 system (as well as other related pathways) is likely not restricted to the adult age, but rather also operates at earlier developmental stages. Thus, studies using models of postnatal under- and overnutrition in female rats indirectly suggested that leptin might also be an important regulator of *Kiss1* and kisspeptin expression during puberty. In rats subjected to underfeeding while nursing, which were leaner and had delayed vaginal opening, a close correlation between low circulating leptin levels at puberty and reduced hypothalamic expression of *Kiss1* mRNA and kisspeptin-positive neurons has been reported.[110] Such an association between early undernutrition, delayed puberty, and suppression of the hypothalamic KISS1 system has been independently confirmed recently in mice[112]; admittedly, however, actual leptin levels were not measured in the later study. Conversely, heavier female rats (due to

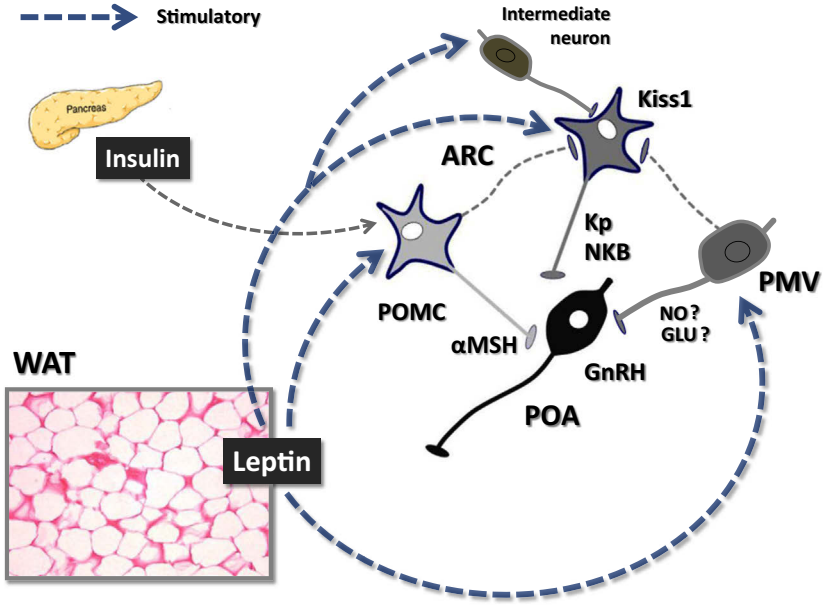

FIGURE 35.3 Potential mechanisms for transmitting leptin actions onto GnRH neurons, as deduced from expression and functional analyses in rodents and other mammals. Since GnRH neurons are devoid of leptin receptors, the actions of the adipose hormone must be conveyed via afferents that are sensitive to leptin effects. These may include (1) POMC neurons, which seem to play a key role in the joint transmission of leptin and insulin effects to GnRH neurons; (2) kisspeptin (Kiss1) neurons, which are sensitive to changes in leptin inputs and operate as major regulators of GnRH neurons; and (3) other neuronal populations, including intermediate neurons (of as-yet-unknown nature), in the vicinity of Kiss1 or GnRH neurons, and neurons in the ventral premammillary nucleus (PMV). Note that fragmentary neuroanatomical and functional data suggest the existence of potential connections between some of these neuronal populations (indicated by dotted-line projections). Such connections may help to explain the fact that leptin appears to operate predominantly in an indirect manner on some of those neuronal subsets (such as Kiss1 neurons). Some of the putative transmitters for these neuronal circuits are depicted. In any event, it is noted that other neuronal populations in the ARC, such as neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons, are known to be sensitive to leptin effects and have been reported to interplay with POMC, GnRH, and (probably) Kiss1 neurons. However, their roles in transmitting leptin actions to Kiss1 and GnRH neurons need further clarification and, for sake of simplicity, are not depicted in this figure. Likewise, other circuits (such as those originating from the lateral hypothalamus) are not represented either. POA: preoptic area; PMV: ventral premammillary nucleus; Kp: kisspeptins; NKB: neurokinin B; αMSH: alpha melanocyte stimulating hormone; NO: nitric oxide; GLU: glutamate; WAT: white adipose tissue.

postnatal overnutrition) showed earlier entry into puberty and displayed higher serum leptin concentrations and *Kiss1* mRNA levels at the hypothalamus.[110] Whether this phenomenon derives from nutritional or leptin changes at early postnatal periods and/or during puberty is yet to be defined.

As a final note to this section, it is emphasized that, in addition to leptin, KISS1 neurons appear to be directly or indirectly sensitive to other major peripheral regulators of the HPG axis, including ghrelin, insulin, and IGF-I. Thus, ghrelin has been reported to suppress *Kiss1* gene expression in discrete hypothalamic (mainly preoptic) areas, an effect that was associated with a significant decline in LH pulse frequency.[126] However, it is not known whether this action is conducted directly on KISS1 neurons or via intermediate afferents. On the other hand, a recent study has documented that congenital elimination of insulin receptors from KISS1 neurons causes a (modest) delay in the timing of puberty in both male and female mice, suggesting a direct action of insulin in the modulation of the hypothalamic KISS1 system.[127] Yet, selective deletion of insulin receptors from KISS1 cells was compatible with preserved fertility, suggesting a dispensable role of direct insulin signaling in the modulation of adult KISS1 neuronal function and reproductive capacity.[127] In addition, IGF-I has been reported to increase *Kiss1* mRNA expression at the AVPV in prepubertal female rats.[128] Yet, blockade of IGF-I receptors did not alter *Kiss1* mRNA levels in this periventricular hypothalamic area in adult female rats.[129]

OTHER PRODUCTS OF KISS1 NEURONS IN THE METABOLIC CONTROL OF REPRODUCTION: THE ROLE OF KNDy NEUROPEPTIDES

In the last few years, it has been recognized that a notable subset of KISS1 neurons in the ARC co-express at least two relevant transmitters involved in the control of the gonadotropic axis, neurokinin B (NKB) and dynorphin (DYN)[1,130]; hence, the term KNDy has been coined to name this neuronal population to recognize such neuropeptide diversity.[130] It must be emphasized, however, that the percentage of ARC KISS1 neurons that co-express NKB and/or DYN seems to vary depending on the species and sex[131];

hence, it is highly plausible that ARC KISS1 neurons that are not KNDy neurons exist. In any event, the relevance of these kisspeptin co-transmitters is emphasized not only by their conserved co-localization in various species, including rodents and primates, but also by the striking reproductive phenotypes of humans with inactivating mutations of the genes encoding NKB (*TAC3*) or its receptor, NK3R (*TAC3R*), which suffer hypogonadotropic hypogonadism.[132–134] Furthermore, stimulatory actions of the NKB agonist, senktide, on LH secretion have been documented in different species,[135–138] while DYN is a well-known inhibitor of GnRH–gonadotropin secretion (see Figure 35.4). All in all, integration of the available neuroanatomical, pharmacological, and genomic data has led to the hypothesis that KNDy neurons in the ARC are key elements in the generation of GnRH pulses, by virtue of their ability to drive a potent stimulatory output signal (kisspeptin) to GnRH neurons, under the reciprocal modulation of NKB (predominantly stimulatory) and DYN (inhibitory).[94,139] Admittedly, however, important functional aspects of this network are yet to be elucidated and notable species differences in the physiological relevance of KNDy neurons in the control of GnRH neurons may exist. For further details on the roles of KNDy peptides in the central control of GnRH neurons, see Chapters 11, 26–28, on the GnRH neuronal network, control of the ovarian cycle of sheep, control of the menstrual cycle, and control of the ovarian cycle of the rat, respectively.

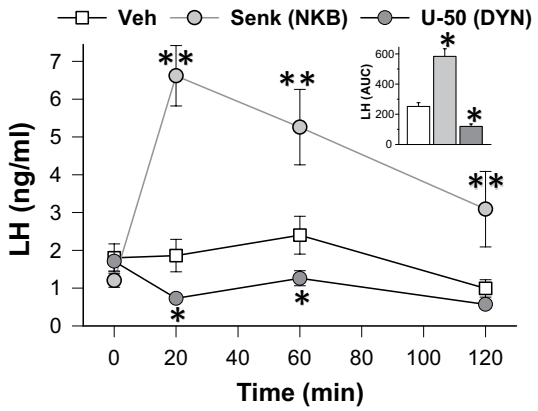

FIGURE 35.4 Effects of intracerebroventricular injection of the agonists of NKB (senktide; 600 pmol/rat) and dynorphin (U-50; 1 nmol/rat) on LH secretion. Adult female rats, subjected to a standard protocol of ovariectomy and physiological estradiol replacement (as described in Ref. 139), were used in these experiments. Saline-injected females (0.9% saline) served as controls. Blood samples were taken before (0 min) and at 20, 60, and 120 min after compound administration. In addition to time-course profiles, the integral secretory responses to the agonists or vehicle were calculated using the trapezoidal rule and depicted in the inset. These hormonal responses illustrate the opposite effects of NKB (stimulatory) and DYN (inhibitory) on GnRH neurons, as indirectly estimated by its surrogate marker, LH secretion. Statistically significant differences are denoted by * ($P < 0.05$) and ** ($P < 0.01$). *Source: Taken from Ref. 139, with modifications, and from our unpublished data.*

In the context of the metabolic regulation of the reproductive axis, recent studies in models of metabolic stress in pubertal animals have demonstrated that, as is the case for KISS1, the hypothalamic NKB system is sensitive to conditions of negative energy balance, so that short-term fasting not only suppressed *Kiss1* mRNA expression in the ARC and AVPV, but also inhibited the hypothalamic expression of the genes encoding NKB and its receptor in pubertal female rats.[140] Importantly, the delay of puberty caused by undernutrition was partially prevented by administration of an NKB agonist (see Figure 35.5), thus supporting the notion that NKB signaling may cooperate with kisspeptins in the metabolic control of puberty.[140] This idea fits well with the proposed model whereby NKB would participate in the central regulation of GnRH secretion by enhancing kisspeptin output to GnRH neurons.[94] As further proof for the sensitivity of NKB pathways to metabolic regulation during puberty, it has been recently demonstrated that feeding on a high-fat diet to female rats from weaning caused precocious puberty onset and increased expression of the gene encoding NKB, as well as *Kiss1*, in the ARC.[109] The role of NKB signaling in the metabolic control of adult reproductive function remains less well characterized, yet certain conditions of negative energy balance and metabolic stress, such as lactation, have been shown to cause a decrease of NKB mRNA and peptide content in the ARC in rats.[42] In addition, the function, if any, of DYN in the metabolic control of puberty and fertility is yet to be elucidated.

Notably, KNDy neurons in the ARC seem to play an important role in the integration of the reproductive and metabolic effects of sex steroids. This has been very recently illustrated by the work of Rance and coworkers, who used a toxic ablation approach to evaluate the consequences of elimination of ARC KNDy neurons on the effects of estrogen upon the negative feedback control of gonadotropin secretion.[141] Ablation of this neuronal population not only altered negative-feedback responses to estrogen withdrawal and replacement, but also abolished the effects of gonadectomy and estrogen supplementation on body weight. This observation would suggest that the integrity of ARC KNDy pathways is indispensable for the conduction of the anorexigenic action of estradiol and would play an important role in energy homeostasis. However, the nature of the KNDy neuropeptide(s) responsible for such a function is yet to be elucidated.

OTHER CENTRAL TRANSMITTERS IN THE METABOLIC CONTROL OF REPRODUCTION

While the recognition of the essential roles of kisspeptins (and related neuropeptides) in the central control of the HPG axis has driven considerable attention to the

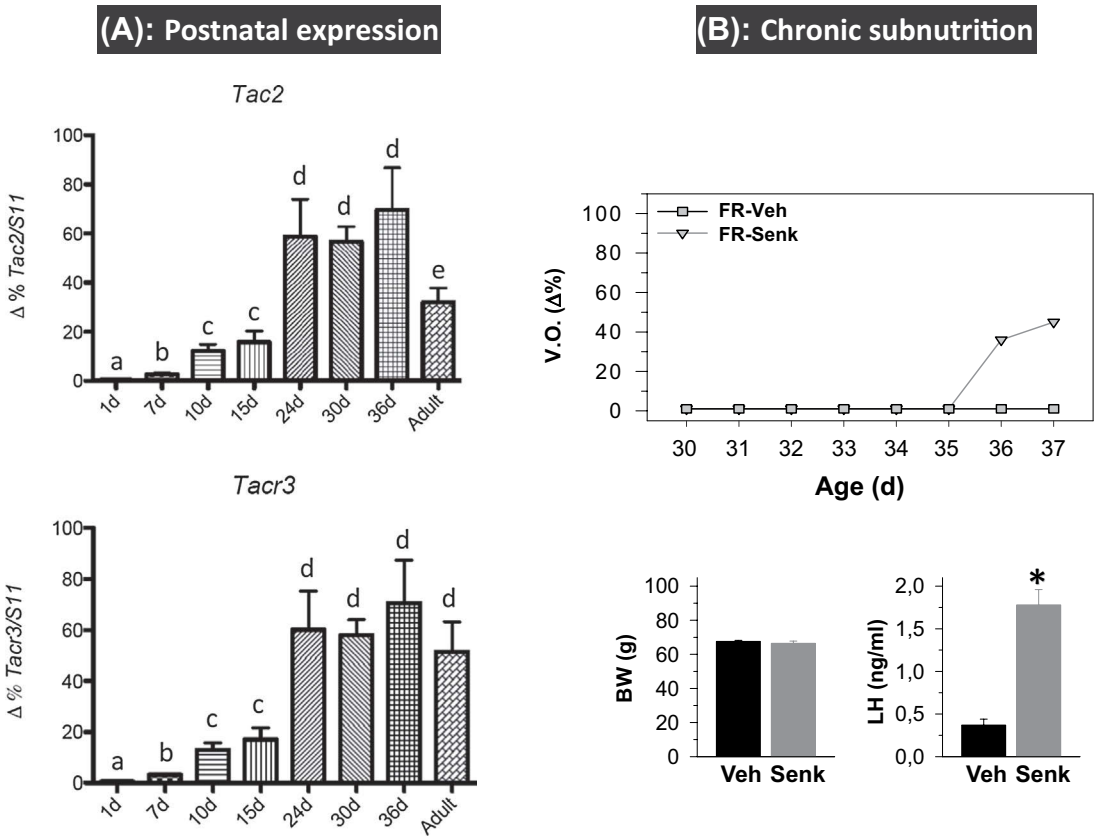

FIGURE 35.5 Characterization of the putative roles of NKB in the control of puberty onset in female rats. The *left panels* (A) show hypothalamic expression levels of the genes encoding NKB (*Tac2*) and its receptor (*Tacr3*) along postnatal maturation and during the pubertal transition. These data illustrate a significant increase in the expression of both hypothalamic genes during the transition between the infantile (<PND21) and juvenile (>PND21) stages. The *right panels* (B) illustrate the effects of repeated central injections of the NKB agonist, senktide, on puberty onset and LH responses in female rats subjected to chronic subnutrition (FR: reduction of 30% in the daily food ration) during the pubertal transition. This regimen of feeding restriction, which induced a drop of ~30% in body weight (BW), was sufficient to cause pubertal arrest, as manifested by lack of vaginal opening (VO) and lowering of basal LH levels in vehicle-treated animals. In this model, repeated central injections of senktide did not change BW but was able to rescue VO in >50% of animals and to induce potent LH responses. Comparison with data from Figure 35.2(B) demonstrate that such effects are remarkably similar to those evoked by kisspeptin replacement in food-restricted pubertal female rats and, together with other results summarized in the section Other Products of KISS1 Neurons in the Metabolic Control of Reproduction: Role of KNDy Neuropeptides, indirectly suggest that NKB cooperates with kisspeptins in the metabolic control of puberty onset. S11: ribosomal protein S11, used as internal control for qPCR assays; PND: postnatal day (age). *Source: Taken from Ref. 140 with modifications.*

analysis of the potential contribution of KISS1 neurons to the metabolic control of puberty and reproduction (see the sections KISS1 Neurons as Central Relays of Metabolic Information: Expression and Functional Studies and Leptin Signaling and KISS1 Neurons: Direct, Indirect, or Independent Effects?), it is clear that other central pathways and transmitters are also involved in this function. In fact, analysis of the available literature strongly suggests that numerous circuits and signals interplay in the dynamic and integrative control of energy homeostasis and reproduction, thus producing a certain degree of redundancy that ensures the best possible adjustment between energy status and reproductive maturation and competence.[4] This redundancy may explain the lack of overt reproductive phenotypes in some models of genetic manipulation of key elements of these regulatory networks, especially when these involve congenital alterations that may trigger developmental compensation. In this section, we provide an account of some of the major transmitters, other than kisspeptins, that have been implicated in the metabolic control of puberty and fertility. Characterization of these central pathways is essential to obtain a complete view on how peripheral metabolic signals impinge on the reproductive brain and ultimately modulate GnRH neuronal function.

As a means of introduction to this section, it is noted that many of the neuropeptides involved in the metabolic control of the HPG axis reviewed herein play an essential role in the central circuits responsible for the regulation of food intake and energy homeostasis.[2] This is well exemplified by the key function of the populations of POMC-cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY)– agouti-related peptide (AgRP) neurons (see the sections POMC and CART Neurons and NPY

and AgRP Neurons) that conduct anorexigenic (appetite-suppressing) and orexigenic (appetite-promoting) actions, respectively.[142] These neuronal populations, located in the ARC, are direct primary targets of peripheral metabolic signals, such as leptin, insulin, and ghrelin, and reciprocally interact to transmit to (and modulate) neurons located in other hypothalamic nuclei, which are ultimately responsible to induce either hunger or satiety.[143] In the same vein, the contribution of other hypothalamic areas, such as the ventromedial nucleus or the lateral hypothalamic area (LHA) (the latter is the source of very potent orexigenic signals, such as melanin-concentrating hormone (MCH) and orexins; see the section LHA: MCH and Orexin Neurons), in the control of feeding is well defined.[142] As described in this chapter, these neuronal populations engage also with the central circuits that ultimately regulate GnRH neurosecretory activity and, hence, qualify as potential integrators for the joint control of metabolism, energy homeostasis, and reproductive function. A summary of the major neuropeptide systems reviewed in this section is provided in Table 35.1.

POMC and CART Neurons

Neurons in the ARC expressing the *POMC* gene are well-known first-order neurons (i.e., those primarily sensing peripheral signals to transmit information to other neuronal populations) in the central control of body weight and energy homeostasis,[144] which are sensitive to the regulatory actions of key metabolic hormones, such as leptin, insulin, and ghrelin. In addition, experimental evidence has documented a potential role of this neuronal population in the metabolic control of the reproductive axis, although elucidation of its precise function in this area has been confounded by the divergent roles of the major POMC products, β-endorphin and alpha-melanocyte stimulating hormone (αMSH), in the control of the gonadotropic axis, as well as potential species differences.[144] On the former, β-endorphin has been reported to inhibit GnRH neurons in different experimental conditions,[145,146] whereas αMSH appears to exert predominantly stimulatory effects, although inhibitory actions also have been reported.[4,144] POMC neuronal terminals appear to form synapses with GnRH neurons.

Interestingly, several transgenic models have been generated that allow delineation of the potential role of POMC neurons in the control of the HPG axis. Thus, mice with genetic inactivation of the *POMC* gene or the genes encoding melanocortin receptors 3 and 4 (*Mc3r* and *Mc4r*, respectively), as major receptors for the central effects of αMSH, develop a metabolic (obese) phenotype and subfertility at adult age.[4,147,148] However, the possibility that perturbation of fertility may derive from persistent obesity in those models cannot be excluded. Interestingly, deletion of leptin receptors from POMC neurons failed to cause any reproductive deficit, which would argue against a major role of this neuronal population in transmitting the reproductive effects of leptin.[149] However, the combined elimination of leptin and insulin receptors from these cells induced follicular abnormalities in the ovary, irregularities of the estrous cycle, and late-onset impairment of fertility in female mice.[150] In double-KO males, subfertility was also detected, despite the fact that testicular weights in adulthood were increased, as were also LH levels.[150] A similar finding was also made in double-mutant female mice, which had elevated LH concentrations. One possible explanation for these observations is that the lack of insulin and leptin receptors in POMC neurons decreased β-endorphin output to GnRH neurons, which would cause hyperstimulation of the gonadotropic axis leading to late-onset partial gonadal failure,[144] a possibility that is yet to be fully tested. All in all, these findings would suggest a role of POMC neurons in conveying the combined effects of leptin and insulin to GnRH neurons. Yet the contribution of the metabolic alterations seen in the double-KO model lacking leptin and insulin receptors in POMC neurons to the reproductive phenotype described here cannot be excluded.[4] To our knowledge, the putative role of POMC neurons in transmitting ghrelin effects to the reproductive brain has not been addressed to date.

POMC and KISS1 neurons have been shown to make mutual contacts in the ovine brain, and an agonist of αMSH enhanced *Kiss1* mRNA levels in the POA of sheep, whereas it decreased *Kiss1* expression in the ARC.[151] In addition, in mice, a subset of KISS1 neurons in AVPV express MC4R.[119] Conversely, kisspeptin has been shown to inhibit *POMC* gene expression at the ARC of sheep,[105] while it directly activates ARC POMC neurons in mice.[152] These observations suggest the potential for interplay between POMC and KISS1 pathways in the metabolic control of GnRH neurons. As an additional note, it is emphasized that ARC POMC neurons also express CART, which may also participate in the control of GnRH neurons.[144] In fact, CART fibers have been found in close apposition to GnRH neurons in rats, and a recent report has described that CART can induce depolarization responses, with a modest increase in the firing rate, in approximately 25% of GnRH neurons in mice.[153] Interestingly, the origins of CART afferents to GnRH neurons seem to be diverse and include not only ARC neurons but also neurons from the PMV.[154]

NPY and AgRP Neurons

The population of neurons in the ARC co-expressing NPY and the functional antagonist of melanocortin receptors, AgRP, reciprocally interact with POMC neurons to modulate food intake and energy homeostasis.[4]

TABLE 35.1 Overview of Different Neuropeptides, with Major Roles in the Control of Food Intake and Energy Homeostasis, Which Participate in the Central Control of GnRH Neurons and the HPG Axis

	Site of Neuronal Cell Body	Hormone Regulators	Response to ↓ Energy and ↓ Leptin	Projections	Effects on Food Intake	Effects on LH Levels	Comments
KNDy peptides NKB Dynorphin	ARC	Leptin	↓	KISS1 (auto) GnRH	↑ (DYN)	↑ (NKB) ↓ (DYN)	Important in KP output and GnRH pulsatility
PMV neurons NO/Glu	PMV	Leptin	↓	KISS1 GnRH		↑	Key for transmission of leptin effects
POMC/CART αMSH CART	ARC	Leptin Insulin Ghrelin	↓	KISS1 GnRH	↓ (MSH) ↓ (CART)	↑ ↑	Project to NPY–AgRP and MCH–orexin
NPY/AgRP NPY AgRP	ARC	Leptin Insulin Ghrelin	↑	KISS1 GnRH	↑ (NPY) ↑ (AgRP)	↑(Y4)/↓ (Y1) ↑/↓	Project to POMC–CART MCH–orexin
GALP	ARC	Leptin Insulin	↓		↑ (alarin*)	↑	KO devoid of effect Receptors unknown
LHA neurons MCH Orexins	LHA		↑	GnRH	↑ (MCH) and ↑ (orexin)	↓ (MCH) ↑ and ↓ (orexin)	Afferents from POMC and NPY
RFRP3	DMH			KISS1 GnRH	↑	↓	Physiological relevance?
SCN pathways NMU–NMS AVP	SCN			KISS1 GnRH	↓ (NMU–NMS)	↑ (NMU–NMS) ↑ (AVP)	Link between rhythms, metabolism, and reproduction?
Nesfatin neurons Nesfatin 1	LHA DMH PVN		↓		↓	↑	Critical in puberty onset Receptor(s) unknown

ARC: arcuate nucleus; PMV: ventral premammillary nucleus; LHA: lateral hypothalamic area; DMH: Dorsomedial hypothalamus; SCN: suprachiasmatic nucleus; PVN: paraventricular nucleus; KP: kisspeptin; NKB: neurokinin B; DYN: dynorphin; POMC: proopiomelanocortin; MSH: melanocyte-stimulating hormone; CART: cocaine- and amphetamine-regulated transcript; NPY: neuropeptide Y; AgRP: agouti-related peptide; MCH: melanin-concentrating hormone; NMU–NMS: neuromedin-U and -S; AVP: arginine–vasopressin; NO: nitric oxide; Glu: glutamate. For further information, see the text. Items without conclusive information are marked as an empty box.

**Alarin is an alternatively spliced form of GALP that shares with it a five-amino-acid stretch at the N-terminus.*

Considering their proven sensitivity to key peripheral metabolic regulators, such as leptin and ghrelin, and the existence of appositions of NPY fibers on GnRH neurons,[155] NPY–AgRP neurons seem to be ideally suited for participating in the metabolic control of reproduction. However, the precise nature and specific mechanisms for this function remain contentious, given the diversity of biological effects, signals, and receptors involved.

The participation of NPY in the central control of GnRH neurons has been well documented, yet both stimulatory and inhibitory actions of this neuropeptide on GnRH–gonadotropin secretion have been reported depending on the pattern of administration, developmental stage, sex steroid milieu, and receptor subtype activated.[8,156] This has been nicely illustrated by electrophysiological studies that documented that while porcine NPY (as an agonist of Y1, Y2, and Y5 receptors) inhibited the firing of 45% of GnRH neurons in mice, a combined agonist to Y1, Y4, and Y5 receptors excited as much as 56% of GnRH neurons.[153] Of note, chronic infusions of NPY have been shown to invariably inhibit the gonadotropic axis,[8] a phenomenon that is compatible with a putative role of NPY in the suppression of the GnRH system in adverse metabolic conditions, such as fasting or subnutrition, in which NPY expression increases. Overall, this inhibitory action seems to be conducted via Y1 (but not Y5) receptors, based on pharmacological and functional genomic studies using the Y1 KO mouse.[157–159] In these studies, deletion of this receptor rendered the mice refractory to the inhibitory effects of caloric restriction on the timing of puberty. Such a predominant inhibitory role of Y1 signaling has been recently confirmed by electrophysiological recordings in mouse brain slices ex vivo[153] and pubertal data from Y1 KO mice.[158] Conversely, predominant stimulatory responses to NPY have been associated with preferential activation of Y4 receptors.[153] Yet, the molecular basis for the observed switch between stimulatory and inhibitory effects of NPY is yet to be fully elucidated.

As was described here for POMC neurons, fragmentary evidence suggests the potential interplay between NPY and KISS1 circuits in the central control of the HPG axis. Thus, studies in sheep recently have documented reciprocal innervations between these two populations.[105] In addition, NPY-null mice have been shown to have lower levels of *Kiss1* mRNA in the hypothalamus and failed to display the expected suppression of *Kiss1* mRNA expression associated with fasting,[98] findings that are suggestive of a stimulatory role of NPY signaling in the control of *Kiss1* expression in vivo. This contention is further supported by a demonstration of stimulatory effects of NPY on *Kiss1* RNA levels in the hypothalamic cell line, N6, in vitro.[98] In turn, sheep studies have shown that kisspeptin increased *Npy* gene expression in the ARC,[105] while in the murine NPY-secreting cell, embryonic mouse hypothalamus cell line 38, kisspeptin was able to increase *Npy* mRNA levels and NPY secretion in vitro.[160] Of note, such a reciprocal stimulatory interaction between NPY and kisspeptin is somewhat counterintuitive, given the fact that conditions of negative energy balance, linked to suppressed *Kiss1* expression, are associated with an increase in the endogenous tone of NPY. In fact, since leptin has been shown to suppress the expression of NPY at specific neuronal populations in the ARC,[161] NPY is not likely to operate as a mediator for the stimulatory effects of leptin on KISS1 neurons but, rather, is likely to act as independent modulator. In any event, it is also known that under certain physiological and experimental conditions, NPY may drive a stimulatory signal to the GnRH system,[162] an effect that may be eventually mediated via kisspeptin afferents.

NPY neurons in the ARC also express AgRP, a functional antagonist of melanocortin receptors, and the inhibitory transmitter, gamma-aminobutyric acid, both of which also may participate in the regulation of GnRH neurons. In line with its expected role as antagonist of αMSH actions, AgRP has been shown to induce inhibitory responses, in terms of LH secretion, in female rats and monkeys.[163,164] Yet, stimulatory effects of AgRP on GnRH and gonadotropin secretion have been reported in male rats.[165] This complexity is further illustrated by recent electrophysiological studies in female mice that demonstrated the ability of AgRP to induce inhibitory or stimulatory responses in subsets of GnRH neurons ex vivo.[153] Of note, a recent report showed that ablation of AgRP neurons in leptin-deficient mice was sufficient to restore metabolic homeostasis and fertility[166]; the latter finding suggests that elimination of a single inhibitory signal—namely, AgRP—activated by the lack of leptin would be sufficient to rescue the reproductive deficit induced by the absence of the adipose hormone. Yet, it remains possible that, at least partially, the foregoing phenomenon may derive from elimination of NPY inputs from the same ARC neurons. To our knowledge, the potential interplay between AgRP and kisspeptin signaling in the control of the reproductive axis has not been documented to date.

PMV Neurons

As described in this chapter, the PMV has now been recognized as a key center for transmitting metabolic information, mainly via leptin signaling, to GnRH neurons. Experimental evidence supporting such a role has been summarized in the section Leptin Signaling and KISS1 Neurons: Direct, Indirect, or Independent Effects? It is emphasized, however, that the pathways and transmitters utilized by PMV neurons to modulate GnRH

secretion have not been totally elucidated; yet evidence has been presented that a subset of PMV neurons, which express functional leptin receptors and innervate GnRH neurons and terminals, co-express the neurotransmitters glutamate and nitric oxide, which may operate as effector signals.[122,167,168] It is noted, though, that PMV neurons are a mixed population and some of the projections to GnRH might not be subjected to leptin–metabolic regulation.[4] It is equally noticeable that some PMV neurons seem to innervate KISS1 neurons, so that their regulatory actions on GnRH neurons might use an intermediate kisspeptin pathway.[4]

GALP Neurons

Another transmitter putatively involved in the central control of metabolism and reproduction is galanin-like peptide (GALP). Within the central nervous system, this neuropeptide is almost exclusively restricted to the ARC, where its expression is inhibited by conditions of metabolic stress and negative energy balance, such as fasting and experimental diabetes, and stimulated by key metabolic hormones, such as insulin and leptin.[3,169,170] In addition, intracerebral injections of GALP have been shown to induce LH secretory responses in a variety of species, including the rat, mouse, and macaque.[169–171] Furthermore, GALP administration was able to rescue puberty onset in food-restricted female rats,[172] as well as to normalize reproductive function in uncontrolled diabetic rats.[173] On this basis, GALP has been suggested to act as a transmitter of metabolic information to GnRH neurons.[169,170] This action might involve its capacity to modulate *Kiss1* expression in the ARC, since central injection of GALP to food-restricted rats, which displayed the expected decrease in *Kiss1* mRNA expression, was sufficient to normalize *Kiss1* mRNA levels.[172] Yet the effect of GALP might not be directly conducted in KISS1 neurons, as intracerebral infusion of GALP apparently failed to induce c-FOS in this neuronal population.[172] The physiological relevance of GALP in the metabolic control of puberty and fertility has been partially questioned by the lack of overt reproductive phenotypes of mouse models with genetic inactivation of either GALP or its major receptors, GALP receptors 2 and 1 (GALR2 and GALR1, respectively).[174,175] Notwithstanding, developmental compensatory events might have taken place in those models of congenital elimination of some elements of the GALP signaling cascade, so that other pathways, including KISS1, may overcome the lack of GALP effects. Hence, although dispensable for the central control of the HPG axis, it remains fully possible that GALP significantly contributes, in cooperation with other pathways, in the metabolic regulation of puberty and reproduction in the adult. Of note, a novel peptide product encoded by a splice variant of *Galp* mRNA, termed alarin, recently has been shown to stimulate feeding and to increase LH secretion by promoting GnRH release,[176] and hence may contribute to the joint control of energy homeostasis and gonadotropic function by GALP-expressing neurons.

LHA: MCH and Orexin Neurons

The LHA is known to play important roles in the control of energy homeostasis and to participate in the neuroendocrine control of reproduction; hence, circuits from the LHA would be well suited for the integrative control of metabolism and fertility.[177] Among the various signals arising from the LHA, two groups of orexigenic neuropeptides, namely, MCH and the orexins—of which two major forms, OXA and OXB, exist—have drawn considerable attention as potential metabolic-reproductive integrators. Concerning MCH, electrophysiological studies demonstrated the ability of this neuropeptide to potently suppress the electrical activity of a subpopulation of septal GnRH neurons in mice,[178] a species where neuroanatomical analyses have shown MCH-positive fibers in the vicinity of GnRH neurons.[178] Moreover, MCH is able to block the excitatory effects of kisspeptin on the septal population of GnRH neurons ex vivo,[178] yet other interactions between the MCH and KISS1 systems (e.g., at the transcriptional level) have not been reported to date. In any event, the available data would support a role of MCH circuits in the integrative control of energy balance and reproduction.

Orexins were first identified as feeding-promoting neuropeptides.[177] In addition, orexins are also known to participate in the central control of a wide array of biological functions, including the regulation of the sleep–wake cycle and arousal states,[179] as well as in the control of various neuroendocrine systems, including the HPG axis.[177] Regarding the latter, hypothalamic GnRH neurons have been shown to express the orexin receptor (OX1R) and to receive direct contacts from orexin fibers.[180,181] In addition, OXA was reported to stimulate GnRH release from rat hypothalamic explants.[182] However, the net effects of orexins on LH secretion appear to be region specific; for example, OXA stimulates gonadotropin release by acting on the hypothalamic POA but suppresses LH by acting in the ARC.[181] These actions of OXA are also dependent on the prevailing sex steroid background. Thus, central injection of OXA stimulated LH release in steroid-primed ovariectomized rats but decreased LH secretion in the absence of ovarian steroids.[183,184] In addition, OXA has been reported to inhibit GnRH-induced LH release by dispersed pituitary cells.[182] All in all, while the above data support a function of orexin pathways in the central control of the gonadotropic axis, the actual physiological relevance of orexins in its regulation by metabolic cues is yet to be fully elucidated.

RF-Amide Related Peptides: RFRP3 and 26/43RFa

RF-amide peptides form a large family of ligands, displaying a distinctive Arg–Phe–NH_2 motif in their carboxyl terminus, which was initially discovered in invertebrates. In mammals, five major groups of RF-amide peptides have been described: kisspeptins, RF-releasing peptides 1 and 3 (RFRP1 and RFRP3, respectively), neuropeptides FF and AF, prolactin-releasing peptide, and the 26/43RFa peptides. Among these, RFRP3, the mammalian ortholog of the avian gonadotropin-inhibitory hormone (GnIH), has drawn considerable interest, as it has been shown to function as a putative inhibitory signal for the HPG axis in different mammalian species,[185–188] acting probably at both central (likely on GnRH neurons) and pituitary levels; further details on the physiological roles of the RFRP3–GnIH system can be found in Chapter 11 on the GnRH neuronal network. Indeed, the fact that both kisspeptin and RFRP3 belong to the superfamily of RF-amide peptides but conduct opposite actions on the HPG axis has led to the proposal that the reciprocal interplay between these two structurally related neuropeptides is essential for the control of reproductive function.[189] While the physiological relevance of such a balance is yet to be fully defined, it is interesting to note that RFRP3 may participate also in the regulation of energy balance, because of its ability to stimulate food intake,[190] and that this action is opposite to that of kisspeptin, which according to some reports inhibits feeding[152]; however, the latter has not been confirmed by independent studies.[95,191] This metabolic dimension of RFRP3 is suggestive of a putative role of this transmitter in linking energy balance and reproduction. To add further strength to this possibility, electrophysiological studies have demonstrated the ability of RFRP3 to blunt the excitatory effects of kisspeptin on POMC neurons.[152]

Of note, the role of RF-amide peptides, other than RFRP3 (and kisspeptins), in the integrative control of metabolism and reproduction has also been addressed, even though the available information is still scarce. In this regard, the additional members of the mammalian RF-amide superfamily, 26RFa and 43RFa, which are orexigenic neuropeptides, have been shown to regulate gonadotropin secretion at hypothalamic and pituitary levels,[192,193] yet their potential interplay with other central transmitters involved in the control of metabolism and reproduction has not been explored to date. In addition, another mammalian RF-amide peptide, the prolactin (PRL)-releasing peptide, has been shown to stimulate LH secretion in female rats,[194] and to cooperate with leptin to suppress body weight and food intake.[195] However, the physiological relevance of this PRL-releasing peptide in the metabolic control of reproduction has not been explored.

Neuronal Efferents from the SCN

The suprachiasmatic nucleus (SCN) plays an essential role in the control of biological rhythms. Because these rhythms are tightly connected with both the function of the HPG axis and metabolic homeostasis, several studies have addressed the reproductive roles of transmitters coming from the SCN. The biological profiles of such transmitters, with proven roles in the control of rhythms and energy balance, make them optimal candidates for linking the seasonal and photoperiodic control of metabolism, puberty, and fertility. This is the case for neuromedin-U (NMU) and neuromedin-S (NMS), which are SCN-enriched peptides with the abilities to suppress food intake and to modulate gonadotropin secretion, acting at central levels, in various physiological conditions.[196,197] Similarly, prokineticin-2 (PK2), which is abundantly expressed in the SCN, has been shown to suppress food intake,[198] to regulate energy balance,[199] and to stimulate LH secretion (author's unpublished data); to what extent these functions are connected and contribute to the metabolic regulation of the HPG axis is unknown. On the other hand, the SCN has been shown to target KISS1 neurons in Syrian hamsters via afferents expressing vasopressin (AVP), a transmitter that seems to play an important role in the generation of the preovulatory surge of gonadotropins in this species.[200] Since AVP has been linked to the control of feeding behaviors and since conditions of negative energy balance (such as 48 h fasting) induced a significant suppression of AVP content in discrete hypothalamic nuclei,[201] it is possible that AVP may provide an additional link between energy status and some important aspects of the function of the HPG axis, such as the preovulatory surge of gonadotropins.

Other Central Pathways: Nesfatin 1 and SF1 Neurons

Other central transmitters also participate in the integrative control of energy homeostasis, metabolism, and the reproductive axis. This is likely the case for nesfatin 1, one of the peptide products of the gene nucleobindin 2 (*Nucb2*) that acts as an anorectic signal in the hypothalamus.[202,203] The relevance of the metabolic function of this peptide is suggested by its expression in hypothalamic areas with key roles in the control of food intake, such as the ARC, the PVN, and the LHA.[203] Very recently, nesfatin 1 has been shown to participate also in the control of female puberty in the rat. Thus, hypothalamic NUCB2–nesfatin 1 expression was reported to increase during the pubertal transition, while conditions of negative energy balance known to perturb puberty, such as chronic subnutrition or short-term fasting, decreased hypothalamic *Nucb2* mRNA and protein levels in pubertal females.[204] In addition, central injection of pmol doses of nesfatin 1 evoked significant

increases of serum LH levels in peripubertal female rats.[204] Conversely, suppression of the endogenous tone of nesfatin 1 in the hypothalamus during the pubertal transition, using an antisense morfolino oligonucleotide (as-MON) against NUCB2, delayed the timing of puberty and lowered LH levels and ovarian weights. Interestingly, studies in adult female rats did not detect gonadotropin responses to central injection of nesfatin 1 at doses that were effective in peripubertal rats, nor did infusion of as-MON against NUCB2 affect spontaneous preovulatory surges of LH and FSH.[204] However, recent preliminary data indicate that, at higher doses, nesfatin 1 can stimulate LH secretion in adult male rats[205] as well as in adult mice (Navarro, Ruiz-Pino, & Tena-Sempere, unpublished observations). Altogether, these features qualify nesfatin 1 as a putative effector for the metabolic regulation of puberty and gonadotropic function.[203] In keeping with this view, preliminary data from my laboratory suggest that blockade of endogenous nesfatin 1 tone, by the use of as-MON against NUCB2, induces a suppression of *Kiss1* gene expression in the ARC and AVPV in pubertal female rats, whereas absence of KISS1R signaling (i.e., in *Kiss1r*-null mice) blocked LH responses to nesfatin 1 (Navarro, Ruiz-Pino, & Tena-Sempere, unpublished observations). The latter suggests a putative interplay between nesfatin 1 and kisspeptin pathways in the central control of the HPG axis, which may be relevant for its modulation by metabolic cues.

Neuronal circuits from the VMH are likely to operate also as a connection center linking energy status and reproduction. In this area, neurons expressing SF1, which are the predominant population in the VMH, seem to be involved in this joint control.[144] SF1 neurons are essential for energy homeostasis, as revealed by the generation of brain-specific SF1 KO mice, which develop obesity due to dearrangement of the VMH.[206] In addition, neurons from VMH have been shown to project to GnRH neurons, and SF1-null female mice display impaired fertility due to inappropriate gonadotropic secretion.[207] The metabolic and reproductive phenotypes of SF1 KOs are roughly similar to those of mice with selective, congenital elimination of ERα from SF1-expressing cells.[79] These findings indirectly suggest a role of estrogen signaling in SF1 neurons at the VMH in the integrative control of energy balance and reproduction. The effectors of SF1 neuronal actions in the regulation of reproduction and their eventual interplay with other central transmitters involved in the metabolic control of the HPG axis are yet to be elucidated.

MOLECULAR MEDIATORS FOR THE CENTRAL CONTROL OF THE HPG AXIS BY METABOLIC SIGNALS

Besides the elucidation of the entire repertoire of peripheral hormones and central transmitters involved in the metabolic control of puberty onset and fertility, considerable attention has been devoted recently to the identification of the molecular mediators linking these sets of peripheral and central signals. These include cellular metabolic gauges that participate in the control of the HPG axis and its regulation by peripheral hormones and metabolic cues, and other molecular cascades that mediate the regulatory effects of different signals (hormones and neuropeptides), which act on key neuronal populations governing the reproductive axis. Altogether, progress in this area has allowed the characterization of the putative roles of several cellular energy sensors, intracellular kinases, ion channels, and transcription factors as mediators for the metabolic control of the major hypothalamic networks governing the reproductive axis. To illustrate the progress in this area, some examples, such as the mammalian target of rapamycin (mTOR), the adenosine monophosphate (AMP)-activated protein kinase (AMPK), the cAMP responsive element-binding protein 1 (CREB1)-regulated transcription coactivator-1 (CRTC1; also termed transducer of regulated CREB1, or TORC1), phosphatidyl-inositol-3 kinase (PI3K), and adenosine triphosphate–sensitive potassium (K-(ATP)) channels, are described in this section.

mTOR has been shown to operate as a cellular metabolic gauge,[208–212] and it seems to mediate some of the effects of leptin in the central control of food intake.[213,214] Experimental evidence obtained in rats strongly suggests that mTOR is also involved in conveying aspects of the regulatory effects of leptin on the reproductive brain, at least in part due to its ability to regulate *Kiss1* expression. Thus, activation of mTOR by central injection of L-leucine, which is one of the major elicitors of mTOR activity, partially reversed the state of hypogonadotropism induced by chronic subnutrition and the resulting low leptin levels.[101] Conversely, persistent blockade of central mTOR signaling delayed the onset of puberty and reduced LH levels; yet this was not caused by a primary effect at GnRH neurons, since they retained their responsiveness to key activators of the gonadotropic axis, such as kisspeptins, which was preserved following mTOR blockade. Importantly, central inhibition of mTOR signaling prevented the positive effects of leptin on puberty onset in a model of caloric restriction.[101] The effects of mTOR inhibition are probably related to a substantial suppression of *Kiss1* mRNA levels in key hypothalamic centers, such as the ARC and, to a lesser extent, the AVPV.[101] Altogether, these observations suggest that leptin may conduct at least part of its regulatory effects on the KISS1 system via the mTOR pathway, which would thereby participate in the metabolic control of puberty onset and fertility (see Figure 35.6). Importantly, these regulatory actions of mTOR do not necessarily occur within KISS1 cells but may take place in intermediate neuronal pathways. In fact, preliminary

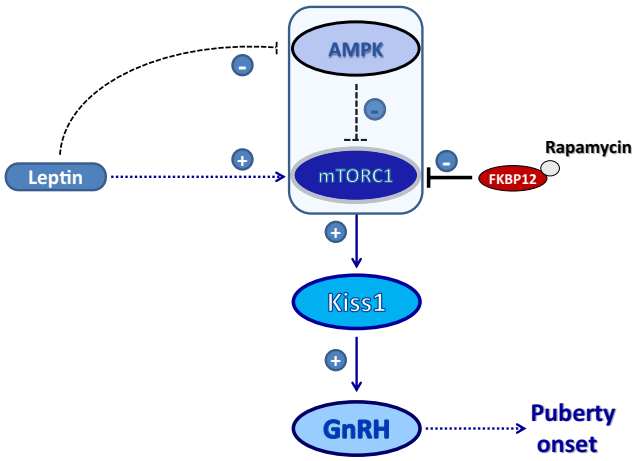

FIGURE 35.6 Tentative model for the potential role of brain mammalian target of rapamycin (mTOR) signaling in the metabolic control of puberty. Leptin, as a key peripheral metabolic signal, is known to modulate hypothalamic mTOR signaling, which mediates part of its regulatory actions in energy homeostasis. In the same vein, experimental evidence suggests that central mTOR may participate in conveying at least part of the actions of leptin on the reproductive brain. This action is likely to involve the regulatory effect of mTOR on *Kiss1* expression, as a major regulatory afferent to GnRH neurons, since blockade of brain mTOR activity not only delayed puberty but also suppressed *Kiss1* mRNA levels in key hypothalamic nuclei, such as the ARC. All in all, the pharmacological and expression data gathered to date strongly suggest that rapamycin-sensitive mTORC1 signaling may operate, in conjunction with other energetic sensors such as AMPK, as a transmitting hub that provides a putative molecular link between peripheral signals and central neuropeptide effectors in the metabolic control of the HPG axis. For further details, see the text. *Source: Taken from Ref. 87, with modifications.*

evidence suggests that a key downstream element of the mTOR cascade, phosphor S6, is not present in KISS1 neurons, therefore suggesting an indirect mode of action of mTOR signaling in the control of the KISS1 system.[108] Of note, mTOR has been suggested to mediate the central metabolic effects of other key hormones, such as ghrelin.[215] Whether the ability of ghrelin to modulate central mTOR signaling is important for its effects on the HPG axis warrants specific investigation.

Another brain fuel-sensing mechanism that might also participate in the central control of the HPG axis is the one involving AMPK, a member of the metabolite-sensing protein kinase family.[216,217] AMPK detects changes in the AMP:ATP ratio and hence in the cellular metabolic state. Thus, in conditions of energy deficit, when ATP is consumed and excess AMP accumulates in the cell, AMPK becomes activated, thus causing the phosphorylation and inactivation of diverse ATP-consuming metabolic cascades. As described for the mTOR pathway, brain AMPK signaling has been suggested to play a pivotal role as a regulator of energy balance and food intake. Activation of AMPK stimulates appetite,[218,219] and leptin has been shown to suppress hypothalamic AMPK activity, whereas ghrelin stimulates it.[220] Interestingly, AMPK and mTOR are mutually regulated (e.g., AMPK inactivates mTOR in different cell systems),[210,221] and hence these two metabolic cell sensors have been proposed to reciprocally cooperate in the central control of energy homeostasis.[213] In this context, it is tempting to hypothesize that this interaction also applies to the metabolic regulation of the HPG axis. While this is yet to be fully proven, indirect evidence would support this possibility, as AMPK activation inhibited GnRH secretion in murine GT1–7 cells in vitro and altered estrous cyclicity, resulting in a modest but detectable change of the interval between consecutive estrous stages in rats.[73,222] In the same vein, our preliminary data suggest that activation of central AMPK signaling delays puberty onset and partially inhibits hypothalamic *Kiss1* expression in the ARC (Roa, Ruiz-Pino, & Tena-Sempere, unpublished observations). All in all, these observations are compatible with a predominant inhibitory role of AMPK pathways in the central control of reproduction, which is in line with its proposed role as a sensor of energy insufficiency and a functional antagonist of mTOR.

Another putative molecular mediator for the metabolic control of the reproductive axis is CRTC1. Evidence for the metabolic and reproductive roles of this transcription factor was provided in 2008, when the phenotypes of CRTC1-null mice were described. These animals were shown to display hyperphagic obesity and infertility.[223] The underlying mechanisms proposed for such a combined alteration of energy homeostasis and reproduction seem to involve the impairment of leptin's capacity to stimulate the expression of the genes encoding CART (which operates as an anorexigenic neuropeptide) and kisspeptins. Leptin has been reported to dephosphorylate and activate CRTC1, which in turn stimulates the recruitment of CRTC1 to *Kiss1* gene promoter. In addition, dephosphorylation of CRTC1 enhanced *Kiss1* gene expression in GT1–7 cells, and CRTC1 overexpression increased *Kiss1* promoter activity.[223] While these observations suggest that CRTC1 may be a molecular node for the integration of metabolic information, and specifically leptin signaling, in KISS1 neurons, this possibility is at odds with the proposed indirect mode of action of leptin on KISS1 pathways (see the section Leptin Signaling and KISS1 Neurons: Direct, Indirect, or Independent Effects?). Moreover, it is noted that an independent report was unable to replicate the consequences of functional inactivation of CRTC1 on mouse fertility.[224] These recent observations therefore warrant the reevaluation of the actual physiological roles of CRTC1 in mediating leptin effects on the HPG axis. Similarly, whether CRTC1 may participate in the central transmission of the reproductive effects of metabolic hormones other than leptin is yet to be defined.

The roles of PI3K have also been evaluated as a potential conduit for the metabolic regulation of the HPG axis. PI3K signaling participates in transmitting the biological effects of leptin and insulin in peripheral metabolic tissues and the brain. An excellent review of the major features of this signaling cascade and its role in metabolism can be found elsewhere.[3] As described in other sections of this chapter, some elements closely related with the PI3K signaling pathway, including ERα, the IGF-I receptor, insulin and leptin receptors, and mTOR, have key roles in the metabolic control of the reproductive brain.[3] Hence, the involvement of PI3K in this function has been addressed by the generation of genetically modified mouse models with selective deletion of key regulatory–adapter or catalytic subunits of PI3K, such as Class-IA, in specific neuronal populations. These studies have revealed that selective elimination of PI3K signaling in POMC or AgRP neurons did not cause an overt reproductive phenotype, therefore arguing against a major, indispensable role of this signaling molecule in mediating reproductive regulatory actions in these neuronal populations. Nonetheless, genetic disruption of PI3K signaling in cells expressing leptin receptors has been shown to prevent leptin effects on key cellular functions in POMC and AgRP neurons in the ARC,[225] as well as in the PMV.[226] Whether PI3K signaling plays a role in the regulation of other neuronal populations, such as KISS1 neurons, which also are sensitive to sex steroid modulation, is yet to be defined.

Because of the known functional properties of leptin receptors in different metabolic tissues, the role of leptin-induced STAT3 signaling in the central control of the HPG axis has been studied.[4] By the use of genetically modified mouse models, it has been demonstrated that, while elimination of STAT3 signaling from the brain results in lack of pubertal maturation and infertility, selective ablation of STAT3 from neurons expressing leptin receptors is compatible with the proper development of reproductive organs and cyclic ovarian function in mice.[227] This suggests that STAT3-independent pathways are activated by leptin in the brain to conduct (at least part of) their effects on the reproductive axis. In addition, these findings point out also that brain STAT3 signaling, activated by factors other than leptin, does participate in the control of puberty and fertility.[4] The nature of such putative regulators of STAT3 is yet to be deciphered.

Finally, the specific role of the K-(ATP) channel, as a mediator of glucose sensing and several neuronal actions of insulin and leptin, in the control of GnRH and LH secretion has been studied by a combination of pharmacological and genomic approaches.[228,229] In this sense, it has been hypothesized that low glucose availability would lead to activation of K-(ATP) channels and, hence, suppressed activity of key neuronal populations governing the HPG axis.[229] In fact, GnRH neurons have been shown to express the K-(ATP) channel subunits, and the blocker of this channel, tolbutamide, depolarized (activated) GnRH neurons and induced GnRH and LH secretion,[228,229] suggesting the potential involvement of K-(ATP) channels in the metabolic control of the HPG axis and its suppression in conditions of low glucose levels. Alternatively, it has also been proposed that low leptin and/or insulin levels in adverse metabolic conditions may lead to reduced K-(ATP) channel activation and, hence, increased activity of key inhibitory afferents of GnRH neurons, such as POMC neurons, which abundantly express this channel.[230] However, fasting-induced inhibition of LH release has been shown to occur in K-(ATP) channel deficient mice, suggesting that this is not an obligatory element for mediating the effects of negative energy balance on GnRH–LH secretion.[229]

REPRODUCTIVE EFFECTS OF METABOLIC HORMONES OUTSIDE THE BRAIN: PITUITARY AND GONADAL ACTIONS

As described in this chapter, a substantial component of the regulatory mechanisms that link metabolism and reproduction takes place at central (mainly hypothalamic) levels by targeting ultimately the GnRH system. However, compelling experimental evidence has demonstrated that metabolic hormones influencing reproductive function, such as leptin, insulin, and ghrelin, also produce biological actions at levels of the HPG axis other than the brain. Because of the focus of this book section, exhaustive review of such *peripheral* actions is clearly beyond the scope of this chapter and can be found elsewhere.[7,22,231] Notwithstanding, some comments on these effects at other levels of the HPG axis will be included in this chapter section in order to illustrate the complexity and diversity of the mechanisms underlying the metabolic–reproductive interactions.

Leptin, insulin, and ghrelin have been reported to act directly at the pituitary, and their actions at this level include the capacity of the three hormones to modulate gonadotrope function and to regulate LH secretion and/or responsiveness to GnRH.[7,8,22] The relative importance of such direct pituitary effects for the metabolic actions of these hormones had been partially neglected, but very recent reports on the impact of selective deletion of leptin receptors in pituitary somatotropes strongly suggest that pituitary cell types, such as GH-secreting cells, can act as metabolic sensors[232]; whether the same applies to gonadotropes is yet to be evaluated. In any event, leptin has been shown to stimulate gonadotropin secretion acting directly at the pituitary level,[233] but the magnitude

of these responses is rather marginal and their metabolic relevance unclear. Likewise, insulin has been reported to directly stimulate LH secretion at the pituitary; this stimulatory effect may be associated with the state of LH hypersecretion seen in patients with polycystic ovarian syndrome (PCOS).[234] The pathophysiological relevance of the direct gonadotropic effects of insulin has been unveiled recently by functional genomics studies showing that selective elimination of insulin receptors from pituitary gonadotropes prevents obesity-induced infertility in female mice.[235] Nonetheless, it is also noted that, in contrast to findings in diet-induced obese mice, pituitary-specific insulin receptor elimination did not cause any detectable alterations of the reproductive axis in basal (lean) conditions.[235] This observation suggests that the physiological relevance of the direct pituitary effects of insulin on gonadotropes, apart from extreme metabolic conditions of obesity, might be modest.

Ghrelin also has been shown to act directly at the pituitary to modulate gonadotropin secretion.[236] The actions of ghrelin at the pituitary appear to involve both stimulatory and inhibitory effects. Thus, ghrelin has been shown to partially suppress GnRH-induced LH secretion by rat pituitaries in vitro. However, direct stimulatory effects of ghrelin on basal LH and FSH secretion by pituitary tissue have been also described in the rat in different physiologic conditions, such as at the various stages of the ovarian cycle and during puberty.[58,236] Such stimulatory action seems to be at odds with the predominant inhibitory effect of systemic ghrelin in the control of gonadotropin secretion. One possibility to reconcile these data is that the above stimulatory effects might derive from the actions of locally produced ghrelin, since gonadotropin stimulation appears to require high ghrelin concentrations,[236] and expression of ghrelin has been reported in the pituitary.[237] However, metabolic regulation of the expression of such pituitary-born ghrelin, and its physiological relevance in linking the energy status and reproduction, are yet to be defined.

Both direct stimulatory and inhibitory actions of leptin and insulin on different aspects of gonadal function have been reported.[7,234,238] Of interest, rodent studies have documented leptin concentrations in the range of those of obese patients can directly inhibit testicular and ovarian steroidogenesis[239,240]; this action was initially considered paradoxical, given the net stimulatory and permissive effect of the adipose hormone in the control of the HPG axis.[238] In any event, such direct inhibitory actions of leptin on some aspects of gonadal function, such as sex steroid secretion, may contribute to the state of hypogonadism commonly associated with conditions of persistent hyperleptinemia, such as morbid obesity.[7] Similarly, as discussed in more detail in Chapter 29 on pathophysiology of the menstrual cycle, hyperinsulinemia has been proposed as a direct contributing factor for ovarian dysfunction and elevated androgens in patients with PCOS.

In the same vein, expression of ghrelin and its canonical receptor, the GHS-R type 1a, has been reported in the testis and the ovary of numerous species, and different studies have documented the ability of ghrelin to directly act at the testicular or ovarian levels to modulate gonadal function.[22] Thus, ghrelin has been shown to inhibit human choriogonadotropin (hCG)-stimulated testosterone secretion by testicular tissue ex vivo, and to partially suppress the expression of genes encoding key steroidogenic factors, such as steroidogenic acute regulatory (StAR) protein, cytochrome P450 side chain cleavage (P450scc), 3β-hydroxy steroid dehydrogenase (3β-HSD), and testis-specific 17β-HSD type III.[241] In line with such putative inhibitory action, ghrelin expression in Leydig cells has been shown to inversely correlate with testosterone levels in men,[242] and ghrelin was reported to suppress the proliferative activity of immature Leydig cells in rat testis.[243] In the case of the ovary, several studies have documented the ability of ghrelin to decrease estradiol and progesterone secretion by cultured human or porcine granulosa–lutein cells.[244–246] In addition, ghrelin may influence additional relevant ovarian functions, including peptide hormone secretion, proliferative activity, and/or apoptosis.[247–249] Notably, other factors with key roles in metabolism and body weight homeostasis, such as resistin, adiponectin, orexin, and nesfatin 1, just to mention some relevant examples, are also expressed and/or act directly in the gonads.[7,250–253] In any event, the pathophysiological relevance of such gonadal expression and actions remains ill defined.

CONCLUSION

In recent decades, a wealth of epidemiological, clinical, and experimental data has substantiated the close connection, long anticipated on the basis of intuitive knowledge, between metabolism, body energy stores, and different aspects of reproductive maturation and function. This association manifests in a wide spectrum of conditions, and has not only important physiological implications but also considerable pathophysiological interest. Indeed, the escalating incidence of human disease or conditions resulting in metabolic disorders, ranging from anorexia to morbid obesity and diabetes, is likely to become (if it is not already) a major threat for human reproductive health. In turn, prevalent reproductive disorders, including PCOS and alterations of puberty, also have a discernible impact on body weight and metabolism, and could hence contribute to the deterioration of the metabolic health of human populations. This evidence has made it mandatory to gain a deeper understanding of the physiological mechanisms

whereby metabolism and reproduction are reciprocally connected.

These efforts have allowed the characterization of the reproductive effects of a large number of peripheral hormones with key roles in the control of metabolism and body weight homeostasis, including leptin, insulin, and ghrelin. Admittedly, this trio is likely to play a major role in transmitting metabolic information to the different levels of the HPG axis. Notwithstanding, it is highly probable on the basis of the available data that additional factors, with important roles in the control of metabolism, also participate in the regulation of various facets of reproductive maturation and function, thereby defining novel regulatory axes whose existence was not documented until very recently.

In the same vein, additional efforts are anticipated in the characterization of the central pathways responsible for transmitting metabolic information to GnRH neurons. The recognition of the inability of GnRH neurons to directly sense changes in key metabolic hormones, such as leptin and insulin, has led to the analysis of different neuropeptide pathways putatively involved in transmitting metabolic information to this neuronal population. In this context, the emergence of kisspeptin as a major regulator of GnRH secretion drew considerable attention to the possibility that specific populations of KISS1 neurons in discrete hypothalamic areas would operate as sensors and transmitters of metabolic cues to GnRH neurons.[1] While the influence of various metabolic hormones, such as leptin and ghrelin, and different conditions of metabolic stress, ranging from short-term fasting to chronic obesity, on the expression and function of the hypothalamic KISS1 system has been thoroughly documented in the last few years, recent evidence has challenged the view that (at least some of) these actions are directly conducted on KISS1 neurons. These studies thus suggest the presence of intermediate afferents responsible for the central transmission of metabolic information to KISS1 neurons, whose nature is yet to be elucidated. It is emphasized, though, that most of the efforts on this front have so far been restricted to the analysis of the actions of leptin, using mainly congenital genetically modified mouse models. Hence, further studies addressing the mode of actions of other peripheral regulators and the generation of even more sophisticated models, such as cell-specific, inducible KO mouse lines, in addition to comparative studies in other species, are needed to fully characterize this phenomenon. In addition, the relative importance of KISS1-dependent versus KISS1-independent circuits in the metabolic control of GnRH neurons needs to be defined. In parallel, it is foreseen that our knowledge of the entire repertoire of central transmitters involved in the metabolic control of reproduction will enlarge in the coming years. This information will also be valuable from a pharmacological perspective, as it will allow us to better characterize the spectrum of biological actions of a number of neuropeptides with potential as therapeutic targets.

The mechanisms whereby metabolic information is integrated within the reproductive circuits have been partially elucidated recently, but our understanding of the precise molecular pathways involved is still incomplete. The mTOR–AMPK duo is likely to play an important role in the central control of energy homeostasis and reproduction, but understanding of the function of AMPK signaling in this context is still incomplete, and the neuroanatomical circuits in which mTOR and/or AMPK are operating are not fully clarified. In the same vein, while the roles of PI3K in mediating the central effects of leptin in the control of body weight and metabolism have been examined by the use of genetically modified mouse models, whether (and, if so, where) PI3K signaling participates in mediating leptin effects on the reproductive brain is yet to be elucidated. Furthermore, the male-biased role of PI3K in the control of GnRH function is intriguing, and its basis and functional implications warrant further investigation. Similarly, whether STAT3 has any role in mediating the reproductive actions of leptin in physiological conditions, as well as the putative function of the transcription factor CRTC1, need further analysis and clarification. Likewise, the molecular mechanisms and primary sites of action of other key metabolic regulators of the reproductive brain, such as insulin and ghrelin, remain ill-defined and will probably attract considerable attention in the future.

In the context of metabolic–reproductive interactions, the effects and mechanisms of action of gonadal steroids on the systems controlling energy balance and food intake have not been completely characterized and will require attention in the future.[78] In this sense, although sex steroids are well-known modifiers of metabolism and body weight, the molecular basis for such a function is, to a large extent, unknown. Anyhow, important progress has taken place recently in the dissection of the primary sites of action of estrogen in the central control of energy homeostasis and reproduction. This work not only confirms the importance of central estrogen signaling in the control of feeding and energy expenditure, a phenomenon with potential implications in menopause-associated metabolic changes, but also demonstrates that distinct neuronal populations play different roles in the integrated control of different aspects of metabolic and reproductive homeostasis. The latter paves the way for the identification of the neurotransmitters and targets involved in such functions.

Finally, while many of the studies addressing the interplay between metabolic signals and the reproductive axis have been conducted using models of negative energy balance, which mimic conditions such as strenuous exercise or anorexia, the impact of obesity, as a highly

prevalent disorder at the other extreme of metabolic disorders, on key aspects of reproductive maturation and function is not so well characterized. Although significant progress has taken place recently on this front (e.g., the characterization of the impact of diet-induced obesity on the hypothalamic KISS1 system), important aspects of this phenomenon remain superficially addressed and warrant further investigation. For instance, whether leptin or insulin resistance to the reproductive actions of these hormones develops centrally in conditions of obesity and, if so, which neuronal circuits are affected are yet to be established. Similarly, the impacts of early metabolic challenges, including obesity, not only on metabolic health but also on key aspects of reproductive function later in life need better characterization. Efforts in these and related areas also will help to better define the pathophysiological basis of common reproductive disorders, such as PCOS.

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CHAPTER

36

Stress and the Reproductive System

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INTRODUCTION

The concept of the *milieu intérieur* was introduced by Claude Bernard in the mid-1800s to describe the principle of dynamic internal physiological equilibrium to sustain the organism in an external environment typified by variability.[1] This is the underlying principle of what would later be called homeostasis, a term introduced by Walter Cannon in the 1930s. He used the physics terminology of strain and stress to describe the homeostatic mechanisms to maintain steady-state conditions. Cannon also incorporated the autonomic nervous system into his model, appreciated psychological impacts, and introduced the term "fight or flight" to describe an animal's response to threats. Hans Selye popularized the term stress (or stressor) for the causative agent in the stress response and critically emphasized that activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenal system is central to the normal physiological response following exposure of an animal to stressors that threaten homeostasis. Indeed, the increase in circulating levels of the glucocorticoids (cortisol or corticosterone) is the benchmark of the stress response.

Selye's doctrine of nonspecificity of stress responses has been challenged. It is now appreciated through the work of Pacák, Palkovits, and many others, who built on earlier descriptions by Cannon, that there is marked heterogeneity of neuroendocrine responses to various stressors with the existence of stressor-specific pathways, neural circuits, and neurochemical signatures.[2] McEwen introduced the term *allostasis*, an extension of the principle of homeostasis, which conceptualizes change in physiological and behavioral systems to attain new set-points that are outside the nonstressed homeostatic range as a stabilizing and adaptive response for survival in the presence of extenuating perturbations. Once these challenges to the system have passed, there is a return to steady baseline levels. Although the allostatic state has a protective function, especially in the short term, if imbalance continues for prolonged periods or additional unpredictable stressors are superimposed, then allostatic overload exceeding the capacity of the individual to cope may occur and pathologies can develop.[3–5]

Stressors have been variously categorized. Perhaps insensibly, for the purpose of this review they are defined as physiological or psychological. Physiological stressors may be metabolic, such as hypoglycemia and fasting, hemorrhagic, thermal, or immunological, to mention but a few. Psychological stressors, on the other hand, encapsulate an element of emotional and cognitive evaluation of the perturbation; common examples include restraint, novelty, noise, and unpredictable and uncontrollable environments. Due to the marked heterogeneity of myriad stressors, overlap in this and other categorizations are common and should be anticipated. For example, it is difficult to separate the physiological and psychological components of food withdrawal or restraint. Furthermore, it is not uncommon, experimentally, to use various stressors in combination to replicate more "natural" scenarios or environmental conditions.

Cameron's laboratory developed a nonhuman primate model of stress-induced amenorrhea, in which cynomolgus macaques are exposed to a combination of mild psychosocial and metabolic stressors (change in social environment plus reduced caloric intake, either with or without exercise)[6] to recapitulate the clinical description of the stressors (psychosocial and metabolic) experienced by women presenting with functional hypothalamic amenorrhea.[7,8] The phenomena of summation and synergism of stressors, including subthreshold stressors, on the reproductive system are implicit[6,9] and could potentially go unrecognized in many experimental settings. Additionally, the impact of environmental factors, often beyond the experimenter's control, such as life history, shipment of animals, or construction work in or nearby animal facilities[10] may also go unrecognized and compromise experimental results.

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In addition to activation of the HPA and sympathetic–adrenomedullary axes, there is a broad spectrum of behavioral responses to stress, including a component of the classical fight-or-flight response. Behavioral responses may be stressor specific. The presence of a predator may cause increased vigilance and freezing behavior to aid survival. Illness or immunological challenge following cytokine or endotoxin administration can result in decreased locomotive activity, exploration, appetite and feeding, and sexual behavior, which enable the animal to cope with the stress of infection and aid recovery. The high-energy demands of stress can also result in a shutdown of key physiological processes that are not required for immediate survival, such as reproduction, which is the focus of this review.

The impact of stress on the gonadotropin-releasing hormone (GnRH) pulse generator, the central regulator of the reproductive system, is reviewed in this chapter, along with a detailed description of mechanisms of action, neural circuits, and neurochemical mediators of different types of stressors, including immunological, metabolic, and psychological stressors. The involvement of the recently discovered kisspeptin signaling system in stress-induced suppression of the GnRH pulse generator is also discussed. Finally, the impact of stress on the ovarian cycle and the preovulatory gonadotropin surge is reviewed. Initially, a brief review of the stress system, including the HPA axis, is necessary as a background for understanding its interaction with the reproductive neuroendocrine axis.

THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Primary Hypothalamic Hormones

The neuroendocrine activation of the HPA axis by stressors involves the secretion of two neuropeptides, corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), from hypophysiotrophic neurons in the parvocellular division of the paraventricular nucleus (PVN) into the hypophysial portal blood, which act in a synergistic fashion on the pituitary corticotrophs to increase pro-opiomelanocortin transcription and adrenocorticotrophic hormone (ACTH) release into the circulation; in turn, this stimulates the secretion of cortisol or corticosterone from the adrenal cortex. The increase in circulating levels of glucocorticoids is key to maintaining carbohydrate reserves, as well as mediating a number of other physiological responses, the actions of which are essential for survival. Such physiological responses include increasing cardiovascular tone, muscle catabolism, and gluconeogenesis in the liver, while at the same time shutting down nonessential physiological activities.

Corticotropin-Releasing Factor

Stressors of various types have been shown to induce *c-fos* and CRF gene expression in parvocellular neurons of the PVN.[11,12] Additionally, stressors result in rapidly increasing levels of CRF in hypophysial portal blood of several species, including rats and sheep.[13,14] Increased levels of CRF in the cerebrospinal fluid of monkeys is evident following acute immunological stress,[15] and persistent elevated levels are found in adult monkeys exposed to early-life adversity[16] and in humans who suffered childhood trauma, (particularly emotional neglect).[17] The histocompatible rat strains Fischer and Lewis provide a comparative model of HPA function, in which the Fischer rats are more reactive to environmental challenges[18] and the Lewis rats are hyporesponsive to myriad stressors, with a significantly lower expression of CRF mRNA in the PVN and lower ACTH and corticosterone release.[19] The administration of type 1 CRF receptor (CRF-R1) antagonists or immunoneutralization of endogenous CRF in a variety of species attenuates or blocks the ACTH and glucocorticoid response to diverse stressors.[1,14,20] Similarly, CRF knockout mice have lower basal corticosterone levels and markedly attenuated, but not completely absent, ACTH and corticosterone response to various stressors, including restraint, hemorrhage, and insulin-induced hypoglycemia.[21,22] Although CRF is the most potent activator of the pituitary-adrenal axis[23] and essential for the normal pituitary and adrenal responses to stressors, other neuropeptides, in particular AVP, are implicated. Additionally, CRF is highly expressed in other brain regions, including the amygdala, bed nucleus of the stria terminalis (BNST), and locus ceruleus, where it increases in response to stress.[24,25]

Arginine Vasopressin

AVP was the first identified secretagogue of ACTH. Although it has an intrinsic capacity to stimulate ACTH secretion, its primary function appears to potentiate the stimulatory effects of CRF,[26] particularly under circumstances demanding sustained activation of the HPA axis.[27] However, it cannot compensate for the loss of CRF in maintaining the normal response to stress. AVP is colocalized with CRF in the parvocellular PVN neurons. Its role in HPA axis activation and the stress response is suggested by increased AVP mRNA levels in the parvocellular neurons[28] and its co-release with CRF[13] into the hypophysial portal blood in response to stress. The synergistic effects of AVP on ACTH release are mediated through the vasopressin V_{1b} receptor on pituitary corticotrophs.[29]

Urocortins

The urocortins (UCN1, 2, and 3) are derived from a common ancestral gene to CRF. They have moderate

sequence homology with one another and with CRF, and they are more potent than CRF in activating CRF receptors, particularly the type 2 receptor (CRF-R2).[30] UCN1 is highly expressed in the Edinger-Westphal nucleus and supraoptic nucleus, but very low levels are seen in the PVN.[31] However, UCN1 participation in stress-related mechanisms is probably derived principally from projections to the PVN and autonomic centers in the brainstem.[31,32] In contrast, UCN2 is highly expressed in the PVN, supraoptic nucleus, BNST, and locus ceruleus[33] and UNC3 is highly expressed in the PVN, BNST, medial preoptic area, and medial amygdala nucleus[34,35]; sites critical not only for stress activation but also control of the reproductive axis.

Administration of UCN1 potently activates the HPA axis, stimulating ACTH and glucocorticoid release in rats and sheep[30,36] and ACTH from rat pituitary cells,[30] which is to be expected from its ability to activate CRF-R1, the receptor subtype expressed by corticotrophs. Nevertheless, UCN-deficient mice exhibit normal basal and stress-induced ACTH and corticosterone release.[37] In contrast to UCN1, systemic administration of UCN2 or UCN3, which are highly selective ligands for CRF-R2, failed to stimulate ACTH or corticosterone release[35] commensurate with an absence of this receptor type in the pituitary corticotrophs.[38] However, unlike central activation of CRF-R1 following intracerebroventricular administration of CRF or UCN, which consistently stimulates ACTH and corticosterone release, central activation of CRF-R2 with UCN2 or UCN3 have yielded inconsistent results, with either no effect[39] or stimulation of ACTH and corticosterone.[34,40] Moreover, intracerebroventricular administration of selective CRF-R2 antagonists has consistently failed to block stress-induced corticosterone release in the rat.[41,42] Additionally, UCN2 or UCN3 null mice show normal stress-induced corticosterone release and negative feedback regulation of the HPA axis, as evidenced by normal poststress recovery of corticosterone levels.[33,43] These data might suggest that CRF-R2 ligands are seemingly unlikely physiologic regulators of the HPA axis. However, CRF-R2 is known to modulate the stress response.

In contrast to CRF-R1 knockout mice, CRF-R2 mutants are hyperresponsive to stress, displaying a more rapid and robust rise in ACTH and corticosterone.[44,45] Additionally, the stress-induced rise in corticosterone is prolonged, suggesting a role for CRF-R2 in the recovery phase of the stress response.[45] A corollary to the postulated hormonal stress adaptive function of CRF-R2 is the enhanced behavioral stress-coping phenotype of the CRF-R2 null mouse.[44,45] Interestingly, CRF-R2 and UCN2 null mice exhibit an altered circadian rhythm in circulating levels of ACTH and corticosterone, with an accentuated rise associated with the awakening response at the light/dark transition.[33] Because the peak and nadir of circadian glucocorticoid release reflects changes in the amplitude of the ultradian pulses, with the largest pulses extant at the awakening transition,[46,47] it is conceivable that the CRF-R2 selective ligand UCN2 may modulate glucocorticoid pulse amplitude. Moreover, UCN2 null mice showed increased AVP expression in hypothalamic PVN magnocellular neurons, which might contribute to the increased peak circadian ACTH and corticosterone levels in these animals.[33] These data raise the possibility that the urocortins, or other CRF-R2 agonists, might modulate circadian HPA axis activity, which could potentially impact on stress responsivity.

Neural Systems Mediating Stress Responses

The HPA axis is governed by a diverse set of neurocircuits that relay stressful information to the PVN, which in turn drives the pituitary-adrenal axis culminating in the release of glucocorticoids. Glucocorticoid receptors are widely distributed in the brain. In addition to the PVN, high levels are expressed in limbic brain areas, including the hippocampus, medial prefrontal cortex, and amygdala, amongst other areas, which are implicated in glucocorticoid regulation of the HPA axis and behavioral responses to stress.[48] Similar to the PVN, administration of glucocorticoids into the hippocampus or medial prefrontal cortex reduces basal and psychological stress-induced glucocorticoid release.[49] Conversely, destruction of the hippocampus or the medial prefrontal cortex exaggerates the glucocorticoid response to stressors in rats[50] and nonhuman primates.[51] Additionally, the forebrain glucocorticoid receptor knockout (FBGRKO) mouse, which has an absence of glucocorticoid receptors in the prefrontal cortex and hippocampus, but retains their presence in the PVN and amygdala, shows a prolonged corticosterone response to acute psychological but not physiological stressors.[52] The stress-inhibitory influences of the medial prefrontal cortex and hippocampus, which are largely psychogenic in nature, are not conveyed by direct pathways to the PVN, but rather indirectly, principally converging onto a common relay in the BNST, which provides an inhibitory gamma-aminobutyric acid (GABA)ergic innervation to HPA effector neurons in the PVN.[53] Thus, the BNST may prove to be a key integrative center for these convergent inputs to restrain psychological stress-induced activation of the HPA axis.

In addition to the negative feedback regulation of the HPA axis, glucocorticoid feed-forward mechanisms act via the amygdala to activate the HPA axis, causing sensitization toward stressors. The central and medial amygdalar nuclei in particular contribute the major amygdalar projections to cortical, hypothalamic, and brainstem regions that regulate autonomic, behavioral, and stress responses[54,55] and play a key role in HPA axis

activation. Lesions of these amygdalar nuclei reduce glucocorticoid secretion in response to stress,[55,56] while their stimulation induces glucocorticoid secretion.[57] Glucocorticoid and mineralocorticoid receptors are expressed in the central and medial amygdalar nuclei.[57] Exposure to stress increases CRF release in the central nucleus of sheep, which depends on glucocorticoid receptor activation.[58] Whether this increase in CRF is from CRF neurons intrinsic to the central amygdala or terminal projections from outside the nucleus, such as the PVN, is not known.[58] Although glucocorticoid infusion into the central amygdalar nucleus does not acutely affect HPA axis activation, selective glucocorticoid receptor deletion in this nucleus reduced CRF expression in the central nucleus and the HPA axis response to stress in mice.[59] Moreover, chronic exposure to glucocorticoids or stress upregulates CRF expression in the central amygdalar nucleus.[60] Continuous overexpression of CRF in this nucleus using a lentiviral vector caused upregulation of CRF and AVP in the PVN and decreased glucocorticoid negative feedback.[61] The central amygdalar nucleus also shows enhanced activation by acute stressors following chronic stress exposure, which suggests that it plays a key role in maintenance of stress responsiveness and stress sensitization.[62] In contrast, the medial amygdalar nucleus may be involved in acute (but not chronic) stress activation of the HPA axis.[63] Additionally, the central and medial amygdalar nuclei respond to distinct stress modalities, with the medial nucleus activated by psychological stressors, including restraint, predator exposure, and social interaction,[50] and the central nucleus preferentially responding to physiological stressors, such as hemorrhage and immunological challenge.[64] In common with the medial prefrontal cortex and hippocampus, the amygdala sends limited direct projections to the parvocellular division of the PVN, with the majority involving subcortical relays including the BNST, medial preoptic area, dorsomedial hypothalamus, and brainstem.[57,65,66] The BNST is of particular significance because it not only receives inputs from inhibitory and stimulatory limbic forebrain regions, but also most hypothalamic and brainstem structures involved in stress-induced control of the HPA axis,[67] which implicates the BNST as an even broader integrative center for regulation of the stress response.

Activation of the brainstem noradrenergic and serotonergic neural systems in the locus ceruleus and raphae nucleus, respectively, along with other brainstem structures, further contribute to regulation of the stress response. The locus ceruleus, containing the principal noradrenergic population in the brain, provides a fountain of innervation to a large portion of the neuroaxis and is implicated in myriad behavioral and physiological functions, including enhanced cognition and memory, vigilance, emotional arousal, and adaptive responses to stress. A wide variety of stressful stimuli activate the locus ceruleus[68–70] and stimulation of this structure elicits HPA axis activation and anxiogenic behaviors.[71] There are extensive reciprocal connections between the noradrenergic neurons of the locus ceruleus and CRF neurons of the PVN, BNST, and central amygdalar nucleus, respectively, providing a neural substrate for feed-forward mechanisms.[70,72] Indeed, functional magnetic resonance imaging in humans has shown that stress-induced noradrenergic activity, a sequelae of locus ceruleus activation, prompts large-scale neural network reconfiguration and enhanced functional connectivity, thus facilitating information exchange between brain regions involved in autonomic-neuroendocrine (e.g. elevated heart rate and cortisol) control and vigilant attention (the latter being a component of the fear reaction in the stress response).[73] There is also extensive literature documenting the importance of serotonergic control of the HPA axis. Serotonergic inputs to the PVN stimulate the HPA axis through both direct and indirect pathways from the raphe nuclei, and lesions of the raphe decrease the HPA axis response to stress.[74,75] Restraint stress increases serotonin (5-hydroxytryptamine, 5-HT) levels in the raphe nuclei and 5-HT_{2A} receptor antagonism attenuates the ACTH response.[74]

In summary, the maintenance of homeostasis in the presence of stress requires a complex range of mechanisms; amongst the most salient is the integrated activation of CRF systems in several key brain areas, including the hypothalamus, BNST, and amygdala, which act with brainstem locus ceruleus noradrenergic and raphe serotonergic systems to coordinate the neuroendocrine and cognitive limbs of the stress response.

Modulation of Stress Responses

Glucocorticoids play a key role in regulating the magnitude and duration of HPA axis activation through myriad negative feedback mechanisms at the level of the pituitary, hypothalamus, and extrahypothalamic sites to restore homeostasis or engage allostasis to minimize the deleterious effects of their long-term exposure. In addition to the classical genomic negative feedback effects of glucocorticoids at the level of the PVN, there is a glucocorticoid-induced fast feedback inhibition of parvocellular CRF neuronal activity that is mediated by nongenomic mechanisms.[76] This rapid feedback mechanism may serve to prevent excessive depletion of hypothalamic and pituitary hormone stores, allowing for repeated stress responses.[77]

Changes in the function of the 5-HT reuptake transporter (SERT), which is critical to the control of 5-HT activity, are also associated with altered stress responsivity. The HPA axis and its feedback regulation are impaired in the SERT knockout mouse, which might account for their increased sensitivity to stress.[78] These

knockout animals are also anxiogenic.[78] Several polymorphisms and mutations have been found in the SERT promoter and coding regions,[79] such as polymorphism in the 5-HT transporter-linked promoter region (5-HTTLPR), which influences the transcription rate of the gene encoding SERT, with the short allele being transcriptionally less efficient than the long allele.[80] Human studies have shown that individuals carrying two copies of the short allele are more vulnerable to stressful events, displaying increased cortisol release in response to psychosocial stress and enhanced vulnerability to psychiatric disease.[81] A similar phenotype is evident in rhesus monkeys with the short/long allele 5-HTTLPR genotype.[82,83]

The HPA axis and its key corticolimbic regulators are particularly sensitive to the developmental programming effects of early life stress, resulting in HPA axis hyperactivity and vulnerability to cardiovascular, metabolic, immunological, neuroendocrine, and psychological disorders in adulthood. Although most attention has focused on the prenatal and early postnatal periods, adolescence (particularly puberty) is a critical developmental period that is also vulnerable to environmental perturbation, resulting in predisposition to psychopathologies and HPA axis hyperactivity later in life.[84–86] Diverse and complex epigenetic mechanisms and genetic liability underlie individual differences in vulnerability and resistance to the developmental programming-induced changes in the stress response. The seminal work of Meaney and colleagues has focused on the influence of variations in maternal care, specifically the frequency of pup licking and grooming in the rat, on the development of behavioral and HPA responses to stress in adulthood.[87] In contrast to the offspring receiving high levels of maternal care, those receiving poor care showed increased vulnerability to stress-induced learned helplessness and enhanced ACTH and corticosterone release commensurate with reduced glucocorticoid receptor expression in the hippocampus, resulting in attenuated negative feedback regulation of the HPA axis.[87] Indeed, the frequency of pup licking and grooming was correlated with the level of hippocampal glucocorticoid receptor expression, revealing a direct relationship between maternal care and the phenotypic development of the offspring.[88] This influence of maternal care on the HPA axis is associated with epigenetic programing of glucocorticoid receptor expression, in particular the methylation status of the glucocorticoid receptor promoter, being hypermethylated in the offspring of low licking and grooming mothers and hypomethylated in those of high licking and grooming dams.[87]

Hypermethylation of the leukocyte glucocorticoid receptor gene was also observed in healthy human adults reporting a history of childhood adversity that was linked to altered HPA axis function.[89] Healthy young adults whose mothers experienced severe stress during pregnancy have enhanced cortisol responses during standard psychological testing.[90] Interestingly, maternal psychosocial stress during pregnancy was associated with a sustained increase in methylation of the glucocorticoid receptor promoter in the blood of their adolescent children.[91] Although there is considerable evidence for familial dysfunction in childhood enhancing stress reactivity,[17,92] interventions that target parental care of high-risk preschool children improve behavior and HPA axis activity.[93] It is of considerable significance that in Maeney's rodent licking and grooming model of maternal care, cross-fostering completely reverses the differences in the methylation state of the hippocampal glucocorticoid receptor promoter.[94] These data support growing evidence suggesting a capacity for the remodeling of epigenetic marks, including DNA methylation, throughout the lifespan of an organism.[87]

Although much attention has focused on epigenetic modifications of genes encoding glucocorticoid receptors,[87] CRF[95] and AVP,[96] which underlie changes in the stress response, it is important to emphasize that other related systems are also susceptible to programing. For example, maternal separation is associated with reduced $GABA_A$ receptor subunit expression in the locus ceruleus and amygdala, affecting behavioral stress responsivity in these rats.[97] Activation of ascending serotonergic systems during pre- and postnatal development regulates hippocampal glucocorticoid receptor expression and the corticosterone stress response.[98] Adverse early life events therefore clearly influence the epigenetic control of multiple regions of the genome in different brain areas to regulate the stress response.

STRESS AND THE REPRODUCTIVE SYSTEM

The reproductive system is under the control of the hypothalamic-pituitary-gonadal (HPG) axis, as described in considerable detail by other contributors to this book (see Chapters 11 and 26–28). Briefly, the function of this neuroendocrine system relies on astonishingly few GnRH neurons (~1000 in higher mammals) in the hypothalamus that synthesize and release their decapeptide into the hypophysial portal blood that stimulates the synthesis and release of the two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary gland. The gonadotropins then act on the ovaries and testes to stimulate gametogenesis and synthesis and release of gonadal sex steroid and peptide hormones. Feedback and feed-forward loops, particularly of the sex steroids, operate at several levels within the HPG axis to control or influence normal function, including pubertal maturation

and ovulation. GnRH and LH/FSH are released in a pulsatile fashion, which gave rise to the concept of the GnRH pulse generator by Knobil[99] (see also Chapters 11, 27, and 28). This neural construct is responsible for the rhythmic activation of the GnRH neurons and the release of the neurohormone from their terminals that eventuate in the pulsatile discharge of LH and FSH into the peripheral circulation.

Despite enormous efforts to identify the neural components of the GnRH pulse generator, they remain elusive. In vitro and ex vivo studies using immortalized cell lines and primary cultures of GnRH neurons, respectively, have indicated an ability of GnRH neurons per se to generate secretory pulses.[100,101] However, electrophysiological recording from single identified GnRH neurons in vitro have proven disappointing,[102] despite the occasional recording revealing a pattern of electrical activity that may reflect a neurosecretory burst underlying a pulse of GnRH secretion,[103] based on comparison with other neurosecretory systems (e.g. oxytocinergic neurosecretory burst underlying pulsatile release of oxytocin during milk ejection associated with lactation[104,105]; see also Chapter 13). The pattern of electrical activity that results in GnRH secretion thus remains a matter of speculation; the classical multiunit activity (MUA) volley recordings pioneered by Knobil are, to the present day, the most remarkable electrophysiological correlates of GnRH pulse generator activity[106,107] (see Figure 36.1).

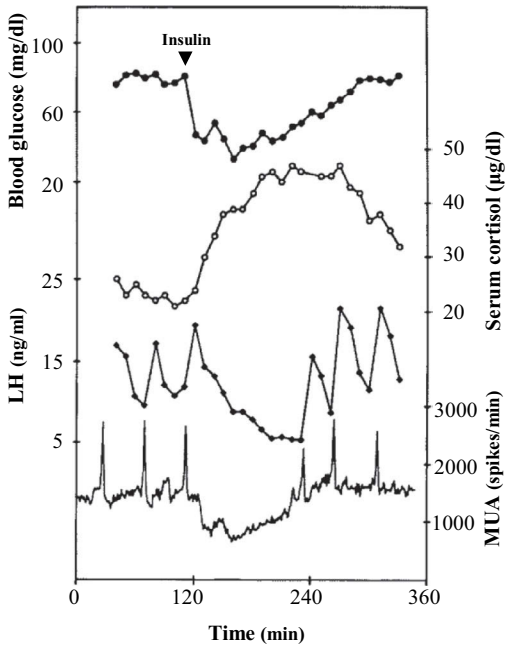

FIGURE 36.1 Effect of insulin-induced hypoglycemic stress on hypothalamic multiunit activity (MUA) volleys and on serum levels of LH and cortisol in a rhesus monkey during the early follicular phase of the menstrual cycle. Note the interruption of GnRH pulse generator activity coincident with the fall in blood sugar and its recovery despite the continued elevated levels of cortisol. *Reprinted with permission from Chen et al.*[107]

Moreover, these MUA volleys are recorded from the mediobasal hypothalamus in rats,[108–111] goats,[112] and rhesus monkeys[106,113] and yet it is only in the latter species that GnRH neurons are located in appreciable numbers in this area. Furthermore, pulsatile LH secretion was not impaired by complete deafferentation of the mediobasal hypothalamus in the rat, which isolates the GnRH neurons cell bodies from their secretory terminals in the median eminence.[100,114] These data generate a conundrum, but indicate that the GnRH pulse generator resides within the mediobasal hypothalamus of various species including rats and monkeys.[100,114,115] Recent discoveries showing a complex intermingling of GnRH dendrites or dendrons as they cascade down toward the median eminence, covered in spines to facilitate synaptic inputs, provides an anatomical architecture to facilitate pulse modulation and potentially pulse generation[116] (see Chapter 11). Moreover, there is emerging evidence[117] that in addition to the many well-established neurotransmitter systems implicated in GnRH secretion, the recently discovered kisspeptin system (particularly the kisspeptin neural population in the hypothalamic arcuate nucleus, known by the acronym KNDy), because of their co-expression of neurokinin-B and dynorphin A, may act as a critical player in the control mechanisms governing pulsatile GnRH secretion.[118–121] Indeed, in the median eminence, kisspeptin fibers densely comingle with GnRH fibers,[122,123] pulses of kisspeptin occur in temporal association with GnRH pulses,[124] kisspeptin antagonists block pulsatile GnRH/LH secretion in several species,[117] and critically intra-arcuate nuclear microinfusion of kisspeptin antagonists suppresses LH pulse frequency in the rat.[125] The role of kisspeptin and KNDy neuron signaling in the control of the GnRH pulse generator is discussed by others in this book (see Chapters 11 and 27).

Inhibitory Effects of Stressors on Pulsatile GnRH/LH Release

It is well recognized that reproduction is suspended or at least delayed when environmental conditions are unfavorable to support the high energy demands of maintaining pregnancy and rearing young. An obvious example of this in the natural world is that most mammals living at temperate latitudes exhibit seasonal variations in reproduction to optimize food availability and other favorable conditions. The critical nature of energy requirements for survival is finely tuned to the stress response with the rising circulating levels of glucocorticoids evident with increasing allostatic load. Allostatic overload, in terms of negative energy balance or metabolic stress, is a cardinal sign of reproductive impairment. States of undernutrition induced, for example, by anorexia or excessive exercise suppress reproduction to preserve energy sources for survival. This fundamental

topic is discussed at length by other contributors to this book (see Chapter 35). However, myriad stressors contribute to the allostatic load and affect reproduction. The complexity of their interactions and underlying mechanisms is gargantuan and, despite enormous progress, still remain to be completely elucidated.

The inverse relationship between the HPA and HPG axes had led to the hypothesis that activation of the HPA system during stress drives the suppression of the reproductive axis. However, the causality and extent of this relationship is far from clear and is all too commonly assumed. Although the pathophysiology of stress-related reproductive failure has not been fully elucidated, the effects of many stressors ultimately converge upon the reproductive neuroendocrine axis to alter secretory patterns of gonadotropic hormone secretion. A major goal is to elucidate the mechanisms, mediators, and sites within the brain and the HPG axis per se, whereby stressors disrupt ovarian and testicular function to reduce fertility. The complexity of stress research is confounded by many factors, including different stressors acting on several different brain areas involving stressor-specific pathways, neural circuits, and mechanisms, coupled with the vast number of stressors used experimentally to mimic the huge diversity of perturbations extant in the natural world. The nature of these challenges (i.e. acute, chronic, or a combination of stressors) affects the response. Developmental stage, age, gender, and endocrine status of the subject are also critical, as are species differences.

Stressors potentially can influence reproductive function at several levels of the HPG axis, but an action on the brain to reduce GnRH pulse generator frequency and hence LH pulse frequency predominates. Not only is the pulsatile nature of gonadotropic hormone secretion an obligatory component of the HPG axis, but critically, the GnRH pulse generator operates within a narrow frequency range, deviation from which results in reproductive failure.[8,126] There is a wealth of evidence from a huge literature spanning many decades that a wide variety of stressors reduce LH pulse frequency in many different species.[1] In contrast to monitoring GnRH pulse generator activity indirectly by measurement of LH pulsatility, there are considerably fewer studies that directly assess GnRH pulsatility for obvious technical reasons. The sheep is the champion animal model from the pioneering work of Clarke,[127] followed by others, including Caraty[128] and Karsch,[129] who collected serial hypophysial portal blood samples for the detection of GnRH pulses. Other large species, including cattle and horses, also avail the opportunity to measure GnRH pulses directly in pituitary and hypophyseal-portal blood.[130,131]

Immunological/Inflammatory Stressors

From the myriad stressors used experimentally, immunological/inflammatory challenges are common. The endotoxin lipopolysaccharide (LPS), which is a major constituent of the outer cell membrane of gram-negative bacteria, induces pathophysiological responses within the body that mimic an acute bacterial challenge and certain aspects of sepsis without causing infection per se. As with infection, endotoxins evoke the release of both peripheral and central cytokines, including interleukin-1 alpha (IL-1α) and IL-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α),[132,133] which are responsible for coordination of the host's immune defense mechanisms. Administration of LPS suppresses LH pulse frequency in a variety of species, including rats,[41,111,134–136] sheep,[13,129,137] goats,[138] cows,[139] and monkeys.[140] In common with LPS, administration of cytokines, such as IL-1α, IL-1β and TNF-α, via intracerebroventricular injection suppresses LH pulse frequency without affecting LH pulse amplitude in rats.[111,132,134,141,142] Although intravenous administration of IL-1β apparently failed to suppress basal LH secretion in rats,[143] intravenous TNF-α suppressed LH pulse frequency without affecting LH pulse amplitude[111]; the latter study used a dose-dependent paradigm that was noticeably absent from the former study. As in the ovariectomized rat,[111] administration of LPS or IL-1α decreases LH pulse frequency but not pulse amplitude in the ovariectomized rhesus monkey.[140] However, levels of FSH were suppressed in the monkey,[144] yet unaffected in the rat.[142] Whether this is related to the longer half-life of FSH remains to be determined. These effects on pulsatile LH secretion in ovariectomized rats and monkeys differ from reports for the ovariectomized ewe, in which both LH pulse frequency and amplitude were affected by endotoxin.[13,129] Moreover, in the ewe, GnRH pulse frequency and amplitude measured in pituitary portal blood are also decreased by LPS.[13] FSH secretion is also suppressed by LPS in the ewe.[145]

In addition to the central mechanisms regulating GnRH secretion, bacterial endotoxin also acts to reduce pituitary responsiveness to GnRH in the ewe.[146] This effect is mediated by pathways that include the synthesis of prostaglandins[147] and cortisol,[148–150] both of which are increased by endotoxin.[149] Whether similar mechanisms operate in other species, such as rats and monkeys, remains to be fully elucidated. Indomethacin does not overcome the suppressive effects of LPS on LH secretion in gonadectomized rats,[151] although it is very effective in restoring LH pulsatility in ovariectomized rats that were adrenalectomized and replaced with corticosterone at basal levels[152]; a model that appears to be particularly sensitive to stress-induced perturbation of the GnRH pulse generator.

Glucocorticoids are not involved in the acute suppression of LH pulses after LPS administration in rats.[153] Indeed, glucocorticoids have been shown to have a protective effect on LH pulsatility under conditions

of infectious stress in the rat, mediated in part by suppression of prostaglandin synthesis in the hypothalamus.[152] Additionally, CRF-R2 antagonism attenuates the LPS-induced suppression of LH pulse frequency without altering the stress levels of corticosterone in the ovariectomized rat.[41] Conversely, CRF-R1 antagonism failed to attenuate LPS-induced suppression of LH pulse frequency, but it reduced corticosterone release as expected.[41] Although the sites of action of CRF-R2 in mediating the inhibitory effects on LPS on pulsatile LH secretion are unknown, neurotoxic lesioning of the central nucleus of the amygdala, which contains a prominent population of CRF neurons,[154] attenuated the response.[136] A role for GABAergic signaling in the medial preoptic area is evident because microinfusion of GABA antagonist into this region blocked the inhibitory response to LPS stress.[155] Further, this effect appears to be $GABA_A$, and not $GABA_B$, receptor mediated.[155]

The putative circuitry leading to the suppression of pulsatile LH secretion by immunological stress in the rat is shown in Figure 36.2.[156] In the rhesus monkey, a nonselective CRF receptor antagonist also blocks the inhibitory effects of LPS on LH secretion without altering the raised levels of cortisol.[157] The inability to prevent the cortisol increase in response to LPS might suggest either an inadequate dosage of CRF antagonist or a mechanism independent of CRF, such as AVP.[157] Although IL-1 receptor antagonists or antisera can completely block the inhibitory effects of IL-1 per se on LH secretion, the inhibitory effects of LPS are only partially blocked in rats and monkeys, confirming, not surprisingly, that other cytokines or factors are involved.[134,140] Indeed, TNF-α antibody also partially blocked the inhibitory effects of LPS on hypothalamic MUA volley and LH pulse frequency in the rat.[111]

Metabolic Stressors

Of the numerous metabolic perturbations that occur naturally or are experimentally induced, the ability of nutritional status, particularly undernutrition, to inhibit the HPG axis is readily inducible, profound, and extant

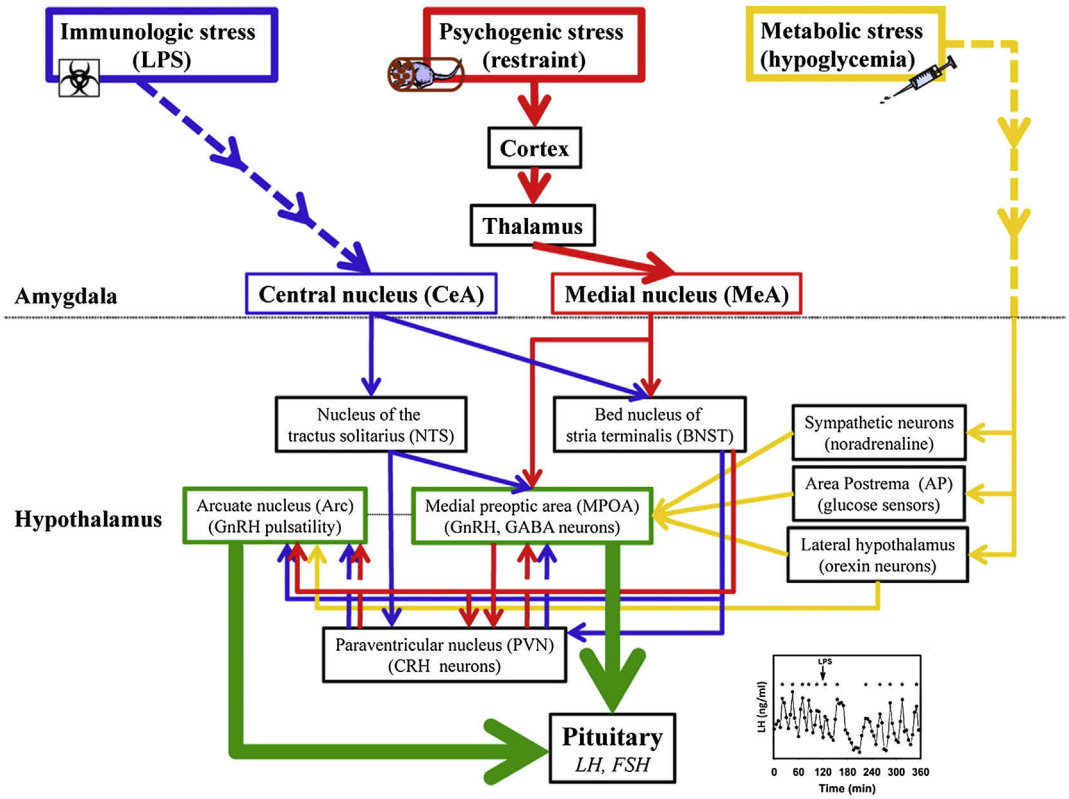

FIGURE 36.2 Putative circuitry leading to the suppression of pulsatile GnRH/gonadotropin secretion by immunologic stress (intravenous injection of bacterial LPS), psychogenic stress (confining the animal inside a restraint tube), or metabolic stress (insulin-induced hypoglycemia). Circuits in part are those directly inferred from the experiments of Lin et al.[136] in which either the medial or the central nucleus of the amygdala was chemically lesioned or the effects of stress on *Fos* expression in these nuclei were measured. The circuits leading to LH pulse suppression by hypoglycemia are unaffected by lesions in either amygdalar location, and the data supporting their routings are derived from other studies as cited in the text. The graphs in the lower part of the figure show the temporary interruption of episodic LH secretion after LPS, restraint or hypoglycemic stress in nonlesioned animals. *Modification from Merriam and Koenig.*[156]

in all species. The impact of nutritional states on reproduction is dealt with by other contributors to this book (see Chapter 35); however, a brief description will be provided here. In men and adult male rhesus monkeys, brief periods of fasting (i.e. 1–2 days) lead to marked decreases in circulating levels of LH, FSH, and testosterone.[158–161] The decrease in LH secretion is consequent to a reduction in LH pulse frequency, with no apparent suppression of LH pulse amplitude, suggesting that fasting affects the hypothalamic GnRH pulse generator rather than a suppression of pituitary sensitivity to GnRH.[158–160] Interestingly, similar short-term fasting (3 days) did not affect the pattern of pulsatile LH secretion in the follicular phase of women,[162] although a modest reduction in LH pulse frequency was observed with 4 days of fasting.[163] However, fasting for 10 days fails to affect pulsatile LH secretion in mildly obese postmenopausal women.[164] Interestingly, refeeding with a high caloric diet had very little normalizing effect on LH pulse frequency within 24 h in women with energetically suppressed LH pulsatility due to a combination of moderate dietary restriction and moderate exercise.[165] These data suggest that women's reproductive neuroendocrine axis is more resistant to caloric alterations than men or male monkeys. Nevertheless, acute fasting suppresses LH pulse frequency in female mice,[166] rats[167] and heifers.[168]

In the male rhesus monkey, refeeding resulted in an immediate increase in LH pulse frequency[169,170] that was proportional to the size of the refeed meal[171]; the type of nutrient in the meal[161] or the meal-induced insulin secretion[172] do not seem to be critical. Additionally, in the monkey suppression of GnRH pulse generator frequency after fasting was found to be a function of the metabolic status rather than the psychological state imposed by withholding food; overfeeding the day before fasting prevented the normal fasting-induced suppression of LH secretion without affecting the agitated behavior associated with food withdrawal.[160] Similarly, intragastric nutrient infusion immediately restored pulsatile LH secretion in fasted animals, although the monkeys continued to display behavioral agitation.[170] It is also of note that cortisol levels are not raised during the fasting period in men, despite profound suppression of LH pulses.[159]

Other forms of metabolic perturbations commonly used include insulin-induced hypoglycemia and competitive antagonism of glucose utilization with 2-deoxyglucose. Insulin-induced hypoglycemia is a highly quantifiable and reproducible stressor that inhibits LH secretion to which animals, including humans, do not habituate.[107,173] A profound reduction in GnRH pulse generator frequency in response to insulin-induced hypoglycemia is observed in the rat,[108,109,174–177] sheep,[178] and rhesus monkey.[107,179,180] Affects on LH pulse amplitude are also reported for the rat[177] and ewe,[178] although not in the monkey.[107,179,180]

An example of the acute inhibitory effects of hypoglycemic stress on hypothalamic MUA volleys and attendant LH pulses in the rhesus monkey is shown in Figure 36.1.[107] These inhibitory effects are readily reversed following restoration on an adequate glucose supply, suggesting that decreased GnRH pulse generator frequency is related to glucopenia and not insulin.[109,173,177–179] Although insulin-induced hypoglycemia is a potent activator of the HPA axis, the inhibition of GnRH pulse generator activity is unlikely to be mediated by cortisol because normal GnRH pulse generator activity was re-established despite the continuing elevation of the steroid[107,179] (see Figure 36.1). As with other stressors, the presence of the ovary or gonadal steroids exacerbates the inhibitory effects of hypoglycemic stress on pulsatile LH secretion.[107,174] Although hypoglycemia clearly suppresses basal LH secretion in men,[173,181] this is apparently not the case in women.[182] However, effects on pulsatile LH secretion in humans have not been determined. Hypoglycemic stress appears not to affect FSH secretion.[173,181,182] In addition to insulin-induced hypoglycemia, acute glucoprivation by 2-deoxyglucose administration profoundly suppresses pulsatile LH secretion in the rat.[152]

Several neurotransmitter systems, including CRF, AVP noradrenaline, opioids, and calcitonin gene-related peptide, are known to mediate the suppressive effects of metabolic stressors on GnRH pulse generator activity.[25,41,174,175,179,183,184] CRF-R2 and not CRF-R1 appears to mediate the inhibitory effect of hypoglycemic stress in the rat.[41] Our knowledge of the neurocircuitry involved in metabolic stress-induced suppression of the GnRH pulse generator is very limited (see Figure 36.2).[156] In contrast to restraint (discussed below) or LPS, the inhibitory effects of hypoglycemic stress on pulsatile LH secretion are not affected by lesioning of the central or medial nucleus of the amygdala in rats.[136] Although CRF antagonism blocks the inhibitory effect of hypoglycemic stress, this is not mediated at the level of the locus ceruleus.[185] However, hypoglycemic stress-induced suppression of LH pulses does involves the area postrema,[175] a key brainstem glucose detection site.[186] The lateral hypothalamic area represents another key site in glucose homeostasis, and its orexin-containing neurons are highly activated during hypoglycemic stress.[2] However, the site of action for orexin-mediated suppression of pulsatile LH secretion remains to be established.[187]

Psychological Stressors

Restraint is a commonly used psychological stressor. Early studies examining the effects of restraint stress on the HPG axis in the rat were limited to the measurement

of mean circulating LH levels, and they reported no change,[188] a decrease,[189,190] or a biphasic pattern with initial elevation and subsequent decline in LH levels[190]; the latter was more commonly observed in the intact male. Effects on FSH secretion were equally variable. More recently, acute restraint stress has been shown to decrease LH pulse frequency without changes in pulse amplitude in the ovariectomized rat,[19,41,110,136,155,191–193] although Nishihara and colleagues observed a reduction in both parameters.[152] Habituation to the inhibitory effects of restraint stress on pulsatile LH secretion can occur rather rapidly in the rat and is related, at least in part, to the level of stress reactivity. The Lewis rat, which is relatively hypoactive to stress, as indicated by reduced synthesis of hypothalamic CRF and secretion of corticosterone, habituated more rapidly to repeated restraint compared with the histocompatible Fischer strain, which is more reactive to stress.[18,19,194] Nevertheless, the habituation of the HPG axis to repeated restraint stress appears not to be related to corticosterone release as corticosterone levels remained elevated.[19]

In male and female rhesus monkeys, restraint in a primate chair suppresses LH pulses,[195] but they readily habituate to this psychological stress,[107] as was also observed in the common marmoset monkey.[9] Again, it seems unlikely that the raised cortisol is causally related to the suppression of LH pulses, because treatment with the opioid antagonist naloxone reversed the effects of restraint on LH secretion without affecting the cortisol levels.[195] In gonadectomized sheep, acute confinement or isolation/restraint stress decreases both LH pulse frequency and amplitude, and the degree of suppression is influenced by gonadal steroid replacement.[196] Transport stress during the follicular phase in the ewe also decreased LH pulse frequency and/or amplitude.[197] A model of psychosocial stress consisting of sequential layering of isolation, restraint, blindfold, and predator cues (barking dog sound and odor)[198] was shown to cause a robust elevation in cortisol and profound decrease in LH pulse amplitude without affecting LH pulse frequency in the ovariectomized ewe.[198,199] This decrease in LH pulse amplitude is a result of two components: firstly, a reduction in pituitary responsiveness to GnRH caused by cortisol acting via the type II glucocorticoid receptor at the pituitary level[198] and secondly, a central effect involving a reduction in GnRH pulse amplitude per se, that was revealed by measurement of GnRH directly in pituitary portal blood, which is independent of cortisol acting via the type II glucocorticoid receptor.[200] However, cortisol does decrease GnRH/LH pulse frequency in follicular phase ewes, an action that is dependent upon the presence of ovarian steroids, particularly estradiol, and sustained (27 h) stress-like levels of cortisol; an acute (6 h) cortisol increase is ineffective in suppressing GnRH pulse generator frequency.[150,201,202] These technically complex studies in the ewe by Karsch and colleagues reveal astonishing detail and insight into physiological mechanisms underlying psychosocial stress and undoubtedly other types of stress induced inhibition of reproductive neuroendocrine function that are difficult to achieve in other species with current experimental techniques.

Another key psychosocial factor is socially induced infertility, which is common in many species including rodents and primates. In many primate species, social status provides a model of psychogenic stress in which subordination is associated with reproductive compromise. Social suppression of fertility in subordinate female common marmosets is mediated by impaired hypothalamic GnRH secretion.[203] Receipt of aggression from a female conspecific reduces pulsatile LH secretion[204] and the preovulatory LH surge in the common marmoset.[9] Similarly, subordinate female rhesus monkeys are hypersensitive to estradiol negative feedback inhibition of LH secretion,[205] which probably accounts for the increased incidence of anovulation and luteal phase insufficiency characteristic of macaques exposed to psychogenic stressors.[206]

Our knowledge of the neurotransmitter systems and neurocircuitry underlying psychological stress-induced suppression of the GnRH pulse generator is equally fragmentary. Nevertheless, both CRF-R1 and CRF-R2 are involved in restraint stress-induced suppression of LH pulse frequency in the ovariectomized rat.[25,41,192] We have shown (unpublished observation) that intramedial preoptic area administration of a nonselective CRF antagonist blocks restraint stress-induced suppression of pulsatile LH secretion in the ovariectomized rat. Although intracerebroventricular administration of UCN2, the selective CRF-R2 ligand, dose-dependently suppresses LH pulses,[192] microinfusion of UCN2 directly into the medial preoptic area failed to suppresses LH pulses, unlike CRF per se, which indicates the medial preoptic area is a probable CRF-R1 site (unpublished observation).

Although CRF signaling in the BNST and locus ceruleus are involved in the inhibitory response to this psychogenic stress, the CRF receptor subtype in these loci has also not yet been determined.[25,41,192] Interestingly, acute restraint evokes LH release in proestrous rats, which is mediated by CRF-R1 in the locus ceruleus.[207] The locus ceruleus receives dense CRF innervation from several regions, including the BSNT,[208] and sends direct noradrenergic projects to GnRH neurons in the preoptic area.[209] Commensurate with the preferential stress modalities conveyed by the medial and central amygdalar nuclei, *vis-a-vis* psychological (e.g. restraint) and physiological (e.g. immunological), respectively, the medial amygdala plays a key role in restraint stress-induced suppression of the GnRH pulse generator that may also involve $GABA_B$ receptor signaling in

the medial preoptic area.[136,155] This contrasts with the role of $GABA_A$ receptors in the medial preoptic area in mediating the effects of LPS[149] (see Figure 36.2).[156]

Impact of Stress on the Hypothalamic Kisspeptin and KNDy System

A major advance in our understanding of the neural mechanisms controlling GnRH secretion came in 2003 with the identification of the critical role for kisspeptins and their receptor, KISS1R (earlier termed GPR-54), in reproductive physiology. Mutations in the *KISS1R* gene are associated with hypogonadotropic hypogonadism in humans[210,211] and similar defects are observed in *Kiss1r* knockout mice.[211] There is now unequivocal evidence that kisspeptins are extraordinarily potent stimulators of gonadotropic hormone secretion in all species studied[110,112,121,212–215]; an effect that, at least in part, involves direct activation of the GnRH neurons that express Kiss1r.[216] The two major hypothalamic populations of kisspeptin neurons that are crucial for the regulation of gonadotropic hormone secretion are located in the preoptic area and the arcuate nucleus (the latter in the KNDy population).[121,217] It is generally accepted that in rats and mice, the preoptic population (which includes those in the anteroventral periventricular nucleus (AVPV)) are critical for controlling the preovulatory gonadotropic surge, while there is increasing evidence that the arcuate population are involved in controlling the pulsatile mode of secretion.[100] In other species, such as sheep, monkeys, and humans, there are fewer kisspeptin neurons in the preoptic area, with the most prominent population residing in the arcuate nucleus, and the latter implicated in both surge and pulsatile modes of GnRH secretion[100,101,121,217–219]; however, the preoptic population is also implicated in surge generation in the macaque and ewe.[219,220]

Because stress-induced suppression of reproduction is associated with reduced gonadotropic hormone secretion, it is reasonable to postulate that the hypothalamic kisspeptin system is implicated in this inhibitory response. Studies using a wide variety of stressors, including LPS, restraint, and insulin-induced hypoglycemia, showed decreased kisspeptin (*Kiss1*) and *Kiss1r* mRNA levels in the arcuate nucleus and preoptic area, which was associated with reduced pulsatile LH secretion in the adult female rat.[135,221] LPS has also been shown to decrease levels of kisspeptin protein in the arcuate nucleus.[222] Under conditions of negative energy balance linked to decreased gonadotropic hormone secretion, such as undernutrition[223] or uncontrolled experimental diabetes[224] *Kiss1* and or *Kiss1r* expression in whole hypothalamus was decreased in rodents. In contrast, undernutrition (72 h fasting) resulted in downregulation of *Kiss1* but upregulation of *Kiss1r* mRNA expression in whole hypothalamus in prepubertal male and female rats,[225] which might explain the increased LH response to exogenously administered kisspeptin in these animals. Similarly, alcohol consumption in prepubertal female rats, which decreases LH secretion and disrupts puberty, suppressed *Kiss1* expression in the preoptic area and arcuate nucleus, but increased *Kiss1r* gene and protein expression in the preoptic area—although not in the arcuate nuclear area,[226] implying an overall reduction of kisspeptin signaling in the latter brain region.

Interestingly, administration of exogenous corticosterone achieving circulating concentrations equivalent to those detected in response to either acute or chronic stressors resulted in a concomitant decrease in *Kiss1* and increase in *Kiss1r* mRNA expression in both the preoptic area and arcuate nucleus in the adult ovariectomized rats.[135] It is tempting to speculate that this differential regulation of hypothalamic kisspeptin and its receptor may underlie the absence of LH pulse suppression in this particular experimental paradigm. Of note is evidence that corticosterone actually plays a pivotal role in maintaining LH pulsatility in the presence of a variety of stressors in the rat.[152] However, the expression profile of *Kiss1* and *Kiss1r* under experimental conditions of hypercorticosteronemia during the estrogen-induced LH surge or across the 4-day estrous cycle, which appear to be sensitive to glucocorticoid suppression,[227] remains to be determined. Additionally, further work is required to determine whether a similar differential regulation of hypothalamic kisspeptin and receptor expression is evident in other species, such as marmoset and rhesus monkeys, which fail to show suppression of LH pulses in response to acute stress levels of cortisol[9,107]; it would also be of interest to compare these data with the effects of chronic hypercortisolemia which does suppress gonadotropic hormone secretion in the monkey.[228] It is similarly unknown how glucocorticoids affect hypothalamic *Kiss1* and *Kiss1r* expression in other species, such as the sheep,[229] which clearly show a suppression of pulsatile LH secretion in response to acute physiological stress levels of these adrenal steroids.

The identification of glucocorticoid receptor immunoreactivity in most (87%) preoptic areas, but few (<3%) arcuate nuclear kisspeptin neurons, in the intact female rat provides evidence of a direct modulation of the kisspeptin system by glucocorticoids, although the physiological implication of the differential distribution of the receptor and its commonality across species remains to be elucidated.[230] Glucocorticoid receptor containing afferents to arcuate nuclear kisspeptin neurons could provide indirect pathways for glucocorticoid modulation of the arcuate kisspeptin system. However, in sheep there is a high percentage (~70%) of co-localization of glucocorticoid receptor with estrogen receptor alpha and progesterone receptor containing

neurons in both the preoptic and arcuate nucleus, which provides indirect evidence for KNDy neurons and preoptic kisspeptin neurons containing glucocorticoid receptors.[231]

Not only are the neural inputs to the preoptic area and arcuate nucleus for stress-induced suppression of *Kiss1*/*Kiss1r* expression undetermined, but few studies have addressed the stress-related neuropeptide systems that may be involved.[232] The classical stress neuropeptide CRF, which plays a pivotal role in stress-induced suppression of the GnRH pulse generator,[25,41,192,233,234] profoundly suppresses *Kiss1* and *Kiss1r* mRNA levels in both the preoptic area and arcuate nucleus when administered by intracerebroventricular injection in the rat.[135] Whether this CRF-induced reduction in *Kiss1*/*Kiss1r* expression directly or indirectly underlies the associated suppression of pulsatile LH secretion remains to be fully determined. The overlapping distribution of kisspeptin[235,236] and CRF-R1 and CRF-R2[24,237] in the preoptic area and arcuate nucleus provides the anatomical substrate for a functional interaction between the CRF and kisspeptin neuropeptide systems. Indeed, it was recently demonstrated that essentially all kisspeptin neurons in both of these hypothalamic sites showed CRF-R immunoreactivity,[230] although the CRF receptor subtype remains to be identified. However, close appositions between CRF fibers and kisspeptin neurons were sparse, leading to the suggestion of a nonsynaptic volume transmission between these signaling systems.[230]

Although CRF signaling in extrahypothalamic areas, including the BNST,[238] locus ceruleus,[185] and medial amygdala,[61] play a critical and stressor-specific role in suppression of the GnRH pulse generator, the link with kisspeptin expressed in these brain regions[239–241] remain to be examined. The inhibitory neurotransmitter GABA is also strongly implicated in stress-induced suppression of the HPG axis.[136,155,242] Moreover, the majority of hypothalamic kisspeptin neurons co-express $GABA_B$ receptors[239] and $GABA_B$ receptors in the medial preoptic area mediate psychogenic stress-induced suppression of pulsatile LH secretion in the rat.[136,155] Although the role for $GABA_B$ receptors in the kisspeptin neurons of the medial amygdala[239] is unknown, it was recently shown that *Kiss1* expression in the medial amygdala, and also the BSNT, were dramatically increased in adult $GABA_{B1}$ knockout mice, which might suggest these extrahypothalamic kisspeptin populations are normally inhibited by $GABA_B$ signaling.[239] However, the role of these extrahypothalamic kisspeptin populations to control of LH is unknown.

Adverse early life environments have been shown to have profound and long-lasting effects on the stress response throughout later life. Neonatal exposure to the immunological challenge LPS delays puberty and decreases *Kiss1* mRNA in the preoptic area in female rats.[243] This reduced *Kiss1* expression persists into adulthood[243] and may contribute to the disruption of estrous cyclicity in these animals.[244,245] Long-term programing of the HPA axis is also evident in these neonatal LPS-treated rats with persistent hypercorticosteronemia and increased stress reactivity in adulthood.[246,247] Early metabolic perturbations similarly affect reproductive function. Models of early-onset obesity, such as a high-fat diet or overfeeding due to small litter size, result in precocious acceleration of the GnRH pulse generator frequency and pubertal activation, as well as high levels of hypothalamic *Kiss1* expression, in the rat.[248,249] Conversely, postnatal undernutrition using a large litter paradigm in rodents resulted in delayed puberty, decreased hypothalamic *Kiss1* expression,[248] and defective development of kisspeptin neural projections that persisted into adulthood, causing lasting deleterious effects on the organization of key hypothalamic neural circuits involved in the control of reproduction.[250]

Despite these many advances, a great deal of further work is required to reveal the complex interaction between the stress system and the regulation of kisspeptin-KISSR signaling in the context of the reproductive axis. Additionally, although the discovery of the kisspeptin system, with its synaptic input to the GnRH neuron, provides the conduit for stress signals to indirectly affect GnRH secretion, preceding theories embraced the notion of stress neural circuits impacting directly as well as indirectly on the GnRH neural network. For example, for GABA, there is a dense GABAergic synaptic input to GnRH neurons in the medial preoptic area.[251] The direct effects of GABA on GnRH neurons are predominantly excitatory, although inhibitory responses are also evident.[252–254] However, intramedial preoptic area administration of GABA suppresses pulsatile LH secretion in vivo.[255] Moreover, stressors or microinfusion of CRF into the BNST or locus coeruleus (both of which inhibit pulsatile LH secretion) activate GABAergic neurons in the medial preoptic area[136,185,238] and, critically, intramedial preoptic area administration of selective $GABA_A$ or $GABA_B$ receptor antagonists block LPS or restraint stress-induced suppression of LH pulses, respectively, in the rat.[155] It is proposed that the primarily suppressive effects of GABA on LH secretion in vivo are most likely explained by indirect influences.[253] Nevertheless, the precise anatomical and functional interactions between the GABA, kisspeptin, and GnRH systems, in particular the identity and relative importance of direct versus indirect inputs to the GnRH neuron per se, in stress-induced suppression of the GnRH secretion—let alone GnRH pulse generator frequency—remain to be established; this is also true for the other key neurotransmitter and neuropeptide systems involved in stress and infertility.

STRESS AND THE FEMALE REPRODUCTIVE CYCLE

Follicular development is dependent on pulsatile LH and FSH stimulation occasioned by the GnRH pulse generator. Although there is a marked increase in GnRH pulse generator frequency during the transition from the late luteal to the early follicular phase of the menstrual cycle in higher primates, including women, that is probably an escape from the inhibiting influence of progesterone. This high frequency of approximately one pulse per hour does not appear to change significantly as the follicular phase progresses and circulating estradiol concentrations rise[113,256,257] (see also Chapter 28). When the estradiol secreted by the developing Graafian follicle exceeds a defined threshold for ~36 h, the preovulatory gonadotropin surge is initiated and ovulation ensues approximately 24 h later—the so-called positive feedback action of estrogen.[258] A similar acceleration of GnRH pulse generator frequency occurs in infraprimate species, including sheep[259] and cattle,[260] although the duration of the follicular phase varies considerably between species. Additionally, the rat shows a clear change in the pulsatile pattern of LH secretion across the 4-day estrous cycle, with a pulse interval of around 46–60 min from metestrus through to the morning of proestrus before the afternoon LH surge.[261] LH pulses are infrequent or absent during estrus, before returning to an approximate circhoral pattern on the morning of metestrus.[261] Without adequate GnRH pulse generator frequency, follicular development might be compromised and insufficient estradiol produced to induce the gonadotropin surge responsible for ovulation and corpus luteum formation. The preovulatory rise in estradiol is critical for the induction of the gonadotropin surge in all species despite important differences in the underlying neuroendocrine mechanisms (see Chapter 33 and the review by Plant comparing monkeys and rodents[262]).

Menstrual and Estrous Cycles: Stress and Ovulation

Disruptive effects of stress on the natural estrous cycle are well established in a wide variety of species, including mice,[263] rats,[100,264,265] sheep,[229,266–268] cows,[269] or menstrual cycle in monkeys[157,270,271] and women.[8,126] Considerable instructive data has been obtained using immunological/inflammatory stress. In the rat, intracerebroventricular infusion of IL-1β for 4–6 days caused a total disruption of the estrous cycle, characterized by persistent diestrous vaginal smears (indicative of low estrogen) and decreased circulating levels of LH and FSH.[151] GnRH gene expression was also markedly suppressed in GnRH neurons distributed between the rostral preoptic area/organum vasculosum of the lamina terminalis and the medial preoptic area in these animals. The percentage of GnRH neurons expressing the c-fos protein in the medial preoptic area during the afternoon of proestrus is also significantly decreased after central administration of IL-1β or peripheral administration of LPS.[151,272] Moreover, GnRH release at proestrus, as measured with a push–pull cannula implanted into the median eminence, is markedly suppressed by IL-1β.[151]

As discussed, cytokines and LPS are known to suppress pulsatile LH secretion in the rat,[41,111,134–136,142] which may contribute to disruption of estrous cyclicity and the preovulatory gonadotropin surge. However, it is also possible that immune challenge interferes with the expression of the LH surge independently of an effect on pulsatile gonadotropin secretion. The underlying mechanism by which an immune challenge could interfere with the surge in the rat remains to be established. Because the LH surge in the rat (in common with the mouse and hamster, but unlike most other species) is critically dependent upon a circadian mechanism,[262,273] immunological stress, in common with other stressors, may interfere either with the interaction between ovarian steroids and the neural substrate in the preoptic area that relays the positive feedback signal to the GnRH neural network or with the circadian signal that times the surge.

The positive feedback action of estradiol that triggers the preovulatory GnRH surge has been shown to be critically dependent on kisspeptin signaling in the AVPV.[274,275] Because endotoxin suppresses *Kiss1* and *Kiss1r* expression in the AVPV in the rat,[135,221] it is not unreasonable to suggest this may be the principal mechanism by which the preovulatory LH surge is disrupted. However, the expression of *Kiss1* in the AVPV neurons and the LH surge are phase-locked to the circadian oscillator located within the hypothalamic suprachiasmatic nucleus, implicating circadian control of kisspeptin in ovulation in rodents, including rats, mice, and hamsters.[276–278] Indeed, an interaction between the suprachiasmatic nucleus and the AVPV kisspeptin neurons via vasopressinergic projections, and a novel preoptic area GnRH gating mechanism to control ovulation has been proposed.[278] Moreover, it was shown that fibroblast growth factor 21 (FGF21), a fasting-induced hepatokine, acts on the suprachiasmatic nucleus to suppress the vasopressin-kisspeptin signaling cascade, thereby inhibiting the proestrous LH surge in mice and providing a novel mechanism for reproductive failure in response to nutritional challenges.[279] However, the relevance of this model to other species remains to be determined.

Mechanistic details have also emerged from studies in sheep, in which the gonadotropin surge is not tightly linked to a circadian system and therefore more akin to primates. In the ewe, endotoxin administration disrupts the estrous cycle by preventing the development of high-frequency LH pulses, which provide the critical drive

for the preovulatory estradiol rise and hence delayed or blocked the LH/FSH surge and estrus.[280] Interestingly, some ewes did not show a suppression of LH pulse frequency; yet, the estradiol rise and LH surge were still disrupted, suggesting that in addition to suppression of LH pulsatility other processes, such as ovarian follicular responsiveness to gonadotropic stimulation, might be impaired.

Immune challenge has been shown to inhibit ovarian estradiol secretion in response to gonadotropins in the rat.[281] In addition to endotoxin acting at the level of the hypothalamus to suppress the pulsatile secretion of GnRH into the pituitary portal blood,[129] it interferes with the ability of the surge-generating mechanism to respond to the preovulatory estradiol signal.[282,283] Unlike its acute suppression of GnRH/LH pulses, which occurs immediately, endotoxin does not acutely inhibit GnRH/LH release at the time of the surge, but rather acts to block the GnRH/LH surge some 10–20 h in advance of the surge,[13,129,283] during the critical estradiol-dependent stage (see Chapter 26 for details) when the steroid must be elevated to activate estrogen-sensitive neurons and the positive feedback signal is transduced to ultimately generate the surge.[284] Endotoxin does not affect the late signal-processing or the surge release stages when elevated estradiol is no longer necessary for successful surge generation.[282] In contrast to endotoxin, transport stress only delays the LH surge when applied immediately before or during the expected time of the LH surge in the ewe,[285] while insulin-induced hypoglycemia can delay the surge at any time before the surge.[286]

Although the detailed mechanisms underlying the role of kisspeptin in the positive feedback effects of estradiol to induce the preovulatory LH surge in the ewe are not fully established, especially the relative contribution of the preoptic area and arcuate kisspeptin populations,[218,287,288] it was shown that LPS-induced suppression of the spontaneous LH surge was associated with a clear decrease in c-fos expression in both kisspeptin neuronal populations.[267] Moreover, there was an increase in the number of CRF-R2 positive cells in the arcuate nucleus and internal zone of the median eminence, which might underlie the LPS-induced inhibition of kisspeptin neurones and disruption of the LH surge.[267] The recent identification of CRF receptor immunoreactivity in arcuate kisspeptin neurons, albeit in the rat,[230] would support this postulate.

Acute psychosocial stress inhibits pulsatile LH secretion in ovariectomized ewes by reducing both GnRH pulse amplitude[200] and pituitary responsiveness to GnRH.[198] Further, a combination of psychosocial stress, such as isolation and nervous temperament through genetic selection, resulting in greater cortisol release, suppresses LH pulse frequency when compared to calm sheep.[268] However, the estrous cycle of the ewe appears to be remarkably resistant to disruption by acute episodes of psychosocial stress, whether applied intermittently during a single follicular phase or repeatedly over the course of several estrous cycles.[289] Similarly, repeated exposure to acute psychosocial stress does not disrupt the estrous cycle in pigs.[290] These data contrast with studies in primates in which psychosocial stress causes prolongation of the follicular phase and luteal insufficiency.[206] Although intravenous infusion of cortisol across the course of the follicular phase (4-days) to achieve concentrations ranging from one third to maximal values induced by isolation stress reduced LH pulse frequency, and delayed or blocked the preovulatory estradiol rise and the LH and FSH surge in the ewe,[201] these plasma cortisol levels are probably more sustained and prolonged than those induced by repeat psychological stress paradigms and reflect more the chronic hypercortisolemia conditions associated with endotoxin stress, which does disrupt the estrous cycle in the ewe.

Functional Hypothalamic Amenorrhea

Clinically recognized forms of stress-induced anovulation and infertility in women include functional hypothalamic amenorrhea (FHA), anorexia nervosa, and exercise-associated amenorrhea. Although it was previously thought that these different forms of anovulation resulted from discrete stressors (FHA: psychosocial; anorexia: nutritional compromise; exercise-associated amenorrhea: excessive exercise), it is now recognized that each of these syndromes develops in response to a combination of psychosocial and metabolic stressors.[6,291] Compared with eumenorrheic women, those with FHA display mild indices of psychological stress including dysfunctional attitudes, difficulty coping with daily hassles, and higher dependence on interpersonal relationships.[292] Additionally, these patients exhibit a high incidence of subclinical eating abnormalities and a propensity to exercise intensively, which adds to their metabolic imbalance.[8] About 30% of women presenting with amenorrhea are diagnosed with FHA and the proximate cause is reduced LH pulse frequency and reduced levels of FSH. Behavioral interventions targeted at alleviating problematic attitudes, such as cognitive behavioral therapy, are not only highly effective at ameliorating FHA but also neuroendocrine and metabolic concomitants, such as hypercortisolism and hypothyroidism.[8]

To study the mechanisms underlying FHA, Cameron and colleagues used a nonhuman primate model in which mild psychosocial stress (novel room with unfamiliar conspecifics) combined with a mild diet, with or without a moderate exercise regimen, led to suppression of reproductive function in some, but not all, animals.[6,291] There is really no equivalent nonprimate model that embraces the nuances of FHA. In her cynomolgus

monkey model, Cameron observed that females were either: (1) highly stress resilient (HSR) and maintained normal menstrual cyclicity when exposed to the combined stressors across two menstrual cycles; (2) medium stress resilient (MSR) with ovulation in the first stress cycle, but not in the second stress cycle; or (3) stress sensitive (SS) with cessation of ovulation as soon as stress was initiated.[6,291] Interestingly, SS monkeys showed lower estradiol levels in the follicular phase and reduced progesterone in the luteal phase of their menstrual cycles prior to any stress exposure, suggesting the HPG axis is less robust than in the more stress resilient monkeys even in the absence of stress.[293] However, the possibility cannot be ruled out that the SS monkeys are slightly stressed under normal husbandry conditions, despite an absence of raised plasma cortisol levels.[294] Moreover, LH pulse frequency was lower in the SS monkeys in the absence of stress.[294] Additionally, animals that developed abnormal menstrual cycles in response to stress (MSR+SS monkeys) showed higher levels of cortisol and suppressed LH pulse frequency during stress exposure, with the latter reversed by CRF-R1 anatgonism.[294]

The failure of the CRF-R1 antagonist to block the stress-induced increase in cortisol secretion might suggest that activation of the HPA axis is not a primary neural mechanism underlying sensitivity to stress-induced anovulation.[294] However, it is difficult to reconcile this with the clinical data where women with FHA showed a reduction in cortisol levels after cognitive behavioral therapy coincident with resumption of ovarian activity.[8] In addition to the endocrine abnormalities and higher basal levels of CRF mRNA in the PVN under nonstress conditions, the SS monkeys also have a greater density of CRF fibers in the central nucleus of the amygdala, locus ceruleus, and serotonergic raphae nucleus, together with a higher noradrenergic output from the locus ceruleus; the latter further increased with moderate psychological and metabolic stress.[270,291,293]

With regard to the serotonergic system, it is downregulated in SS monkeys, as indicated by lower gene expression of tryptophan hydroxylase 2, the rate-limiting enzyme for 5-HT synthesis, SERT, 5-HT-receptor, subtype 1A in the dorsal raphe nucleus compared with HSR animals.[291] Interestingly, although polymorphism of the 5-HTTLPR (in particular, a preponderance for the short allele) is associated with increased sensitivity to stress and anxiety in humans and rhesus monkeys,[81–83] the short allele was not observed in the SS cynomolgus.[295] There is also reduced 5-HT innervation of the locus ceruleus in SS monkeys, the source of which might be the raphe.[270] Importantly, selective serotonin reuptake inhibitors (SSRI) increase peak estradiol and progesterone secretion only in SS monkeys, suggesting that adequate central serotonergic drive to the HPG axis is critical and SSRI's may be an effective treatment for FHA.[291] In the hypothalamus of SS monkeys, there is an upregulation of 5-HT_{2A} and 5-HT_{2C} receptors and an increase in GAD67 gene expression, which are inhibitory to the HPG axis.[296]

There are considerably less data on men. Stress related to prolonged military endurance training is associated with reduced plasma levels of gonadotropins and androgens and raised cortisol.[297] However, it is difficult to separate the relative contribution of psychological and metabolic stressors under these conditions and caloric deficits are known to readily suppress pulsatile LH secretion in men.[159] Nevertheless, anticipatory psychological stress suppressed LH and testosterone levels in men.[298]

CONCLUSION

We have a limited grasp of the neural mechanisms underlying stress-induced reproductive failure. Given the myriad stressors, the complexity of the neurocircuitry, and their stressor-specific nature, coupled with species differences—to mention just a few of the variables to consider—it is little wonder. Nevertheless, at the center of the neuroendocrine component of the reproductive system lies the GnRH pulse generator, which is unequivocally affected by stress and a reduction in its operational frequency is at the core of stress-related infertility. Less well characterized is the stress-induced reduction in GnRH pulse amplitude or LH pulse amplitude that may also impact on reproductive function.

Kisspeptin signaling has provided a recent focus on the neural mechanisms involved, not only in GnRH pulse generation but GnRH surge generation, although we have only begun to scratch the surface of the stress neuropeptide-kisspeptin interactions. Peripheral components of the HPG axis (i.e., the ovary and testis,) and the autonomic nervous system have not even been touched on in this review; the reader is directed to the elegant and extensive review by Ferin in the previous edition of this book.[1]

There are many unresolved questions concerning the effect of stress on reproduction. One of the greatest challenges will be a more complete understanding of the complex neural circuitry underlying the suppression of gonadotropic hormone secretion in response to various stressors. Their identification will help reveal the critical neurotransmitters or neuropeptides involved, thereby facilitating the discovery of novel therapeutics for stress-related reproductive disorders. However, we can anticipate significant redundancy in the neural circuitry, as evident in part by the common observation of partial blockade of the stress response with neuropeptide antagonism, nuclear lesions, or gene knockdown or silencing. A greater understanding

of the neural basis of individual variation or vulnerability to stress is essential. Finally, identification of the neural construct underlying the GnRH pulse generator would greatly facilitate elucidation of the pathophysiology of stress-related reproductive neuroendocrine disorders.

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CHAPTER

37

Aging and Reproduction

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INTRODUCTION

Overview

The capacity to reproduce, more than almost any physiological process, undergoes enormous functional change across the life cycle. In the case of placental female mammals, the physical and psychological ability to maintain pregnancy, lactate, and care for offspring must be achieved if reproduction is to be successful. Reproductive processes must be further coordinated with external environmental forces such as food availability, social factors, and freedom from stressors, as well as internal factors such as disease states, hormonal deficiencies/excess, and immune challenges. In the longer term, reproductive competence is coordinated with chronological age, being attained at the onset of puberty, following an often extensive postnatal developmental period, and lasting through adulthood. With advanced aging, there are further reproductive changes, typically a loss of reproductive capacity in the female with partial or complete reproductive senescence. In contrast, aging effects on the male reproductive axis are much less pronounced, and reproductive capacity is typically sustained until very late in life.

Reproductive aging refers specifically to germ cells and the hormone-producing cells that support them, both within the gonad and at the hypothalamic and pituitary level. During the reproductive aging process, each level of the hypothalamic-pituitary-gonadal (HPG) axis undergoes alterations in structure, function, and synthesis/release of hormones. In addition, ovarian and testicular hormones exert feedback actions on the hypothalamus and pituitary, which may also change with aging. While reproductive aging occurs within the broader context of somatic aging, here we will focus on how the reproductive systems of females and males change with age, discussing physiological functions, hormones, and underlying mechanisms. For more detailed discussion of the GnRH neuronal network, the pituitary gonadotropes, sexual differentiation, the neuroendocrine control of puberty, and ovarian cyclicity the reader is referred to Chapters 7, 10, 11, 28, 32, and 33.

Hypothalamic-Pituitary-Gonadal (HPG) Axis

The control of reproduction across the life cycle involves three levels of regulation, each playing a unique and critical role. In the hypothalamus, a small group of a few thousand neurons that produce the neuropeptide gonadotropin-releasing hormone (GnRH)[1] provides the primary driving force upon the reproductive system through most of life. This small neural population, whose cell bodies are localized in anterior hypothalamic regions (rodents and primates) and the basal hypothalamus (primates), project neural processes to the median eminence/infundibulum at the base of the hypothalamus and just above the anterior pituitary gland. The GnRH decapeptide is released in pulses with intervals of 30 min to several hours, depending upon species, and in the case of females, upon menstrual cycle stage. From the median eminence/infundibulum, GnRH diffuses into the portal capillary system that transports it and other hypothalamic releasing/inhibiting hormones to the anterior pituitary gland to act upon target cells.[2] Specific to HPG function, a subpopulation of anterior pituitary cells called gonadotropes, which express GnRH receptors and

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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that synthesize luteinizing hormone (LH) and/or follicle-stimulating hormone (FSH), respond to the GnRH signal with release of LH and FSH into the general circulation. From there, LH and FSH act upon their receptors in the ovary or testis to affect the primary gonadal functions of gametogenesis and steroidogenesis. The steroid hormones, particularly estradiol and progesterone in females, and testosterone and estradiol in males, have powerful actions on reproductive and nonreproductive tissues and exert feedback control of hypothalamic GnRH neurons and pituitary gonadotropes. The gonads also secrete protein hormones that are involved in reproductive processes. Inhibin B in males and inhibin A and inhibin B in females play important roles in the selective suppression of FSH release from gonadotropes.

The relationships among the hypothalamus, pituitary, and gonad change throughout the life cycle. Gonadal development in the embryo begins early in gestation and occurs independent of the central components of the reproductive system, which develop later in gestation. Anti-Mullerian hormone (AMH) production from male testes leads to regression of the Mullerian system (Chapter 7). The testes also produce relatively high levels of testosterone that are critical for masculinization of the reproductive tract and other tissues, in response to maternal chorionic gonadotropins in primates. In contrast, the fetal ovaries are relatively quiescent. The hypothalamus and pituitary develop later in gestation, with hypothalamic GnRH neurons detectable approximately halfway through gestation. Shortly before/after birth, the gonads actively synthesize steroid hormones, the testis more so than the ovary, resulting in further reproductive developmental changes and sexual differentiation of the brain, the latter well-defined in rodents albeit less clear in primates (Chapter 47). In humans and nonhuman primates, infancy is characterized by robust pulsatile GnRH release, elevated levels of gonadotropins, and gonadal steroid secretion, with steroid secretion being more pronounced in males than females (Chapter 32). Following this "mini-puberty of infancy," gonadotropin release and gonadal hormone production are markedly decreased until the onset of puberty.

The pubertal process is well accepted as being governed by the activation of GnRH neurons by converging central nervous system inputs from other neurons and glia that signal through the neuropeptide, kisspeptin, as well as other neurotransmitters and neuropeptides. Steroid hormone feedback to the hypothalamus and pituitary also matures during this early phase of reproductive life. It is now well accepted in females that stimulatory central drive from a network of hypothalamic inputs to the GnRH system, together with a diminished drive from inhibitory neurotransmitters, are associated with puberty (see Chapters 30–32). These neural networks continue to play this role in adulthood; however, age-related changes in reproductive age processes are still relatively poorly understood.

The remainder of this chapter will focus on the sex-specific, age-related changes in the reproductive axis that occur from adulthood into aging and discuss the relationships between the hypothalamic, pituitary, and gonadal components of the axis. As was the case for our understanding of the neuroendocrine and gonadal physiology of puberty and normal reproductive function, animal models of reproductive aging provide an important complement to studies in men and women. Not only can animal models be used to address mechanistic issues that cannot be approached in the human, but they also emphasize both the common features and the unique reproductive strategies that have developed across species to ensure survival.

Historical Perspective

The age-related loss of menstrual function in women has been noted throughout recorded history. However, it was not until the late 1700s that discussion of the etiology of these changes transitioned from mystical to physical terms with John Leake's proposal that cessation of menses was caused by vascular comprise (for review, see Haney[3]). In 1952, the seminal studies of Block[4] documented the dramatic decrease in the number of follicles within the ovary as a function of age, establishing ovarian aging and the loss of steroid hormones as critical to the loss of reproductive function and vaginal bleeding. Changes in gonadotropin secretion after menopause and in menstrual cycles of older women were documented in the 1970s.[5] For many years, the selective rise in FSH on day 3 of the menstrual cycle in older women was the only clinically available marker of potentially declining fertility in such subjects Studies in the 1980s confirmed that this rise in FSH was due to the loss of ovarian secretion of both estradiol and the inhibins, particularly inhibin B.[6,7] Since that time AMH has been established as an unexpected marker of the number of ovarian follicles, and AMH and antral follicle count have been used as prognostic markers in infertility programs (for review see Broekmans et al.[8]). In 2001, the first Stages of Reproductive Aging Workshop (STRAW) utilized the findings from important cohort studies of women across the menopause transition to derive a codified description of reproductive stages across the lifespan, based primarily on changes in menstrual bleeding patterns and changes in FSH levels.[9] This staging system provided a critical scaffold for further studies and was updated a decade later (STRAW+10) to incorporate the results of longitudinal studies across the menopause transition and studies of fertility in older women,[10] as described below.

Perhaps the most important study in rodents is that of Krohn et al.,[11] in which ovaries were transplanted from

aged to young ovariectomized animals. These studies demonstrated that ovaries from old donors undergo folliculogenesis and ovulation in the young hosts, suggesting that reproductive failure with aging in rodents is not limited by the ovary, and paved the way for future studies on identifying the changes in the hypothalamic-pituitary axis that are responsible for the cessation of reproductive function during aging.

A reduction in testosterone levels in the spermatic veins of elderly men was first reported more than five decades ago.[12] However, subsequent studies using peripheral blood samples produced conflicting results due to small sample sizes as well as differences in the health of the cohorts studied, the methodology used, and the timing of the blood sampling.[13] It has only been in the last two decades that large epidemiologic studies have clarified the magnitude of the change in testosterone with aging[14–17] and subsequent detailed physiologic and histologic studies in both human and primate models have implicated alterations at both the hypothalamic and testicular levels that account for these changes.

FEMALE REPRODUCTIVE AGING

Reproductive Function in Normal Reproductive-Aged Women

Within 2–4 years of the first menstrual period, regular reproductive cycles are established in normal women. Cycles between 25 and 35 days (from the first day of menses in one cycle to the first day of menses in the subsequent cycle) are considered normal,[18] although cycle-to-cycle variability in an individual woman is considerably smaller (±2 days). Variability in overall menstrual cycle length both between and within women is largely due to differences in follicular phase length with luteal phase length remaining relatively constant.

The hormonal interactions between the hypothalamus, pituitary, and ovary, with the endometrium as a gonadal steroid end organ for reproduction, are tightly orchestrated and precisely timed (for review see Hall et al.[19]) (Figure 37.1). Estradiol is responsible for endometrial proliferation in the follicular phase, while both estradiol and progesterone are required for luteal phase preparation of the endometrium for implantation if conception occurs. If pregnancy does not occur, the loss of hormonal support of the endometrium that accompanies the decline in function of the corpus luteum results in endometrial shedding or menses, which provides an external marker for the beginning of a new cycle.

Changes in ovarian secretion of progesterone and estradiol modulate the frequency and/or overall

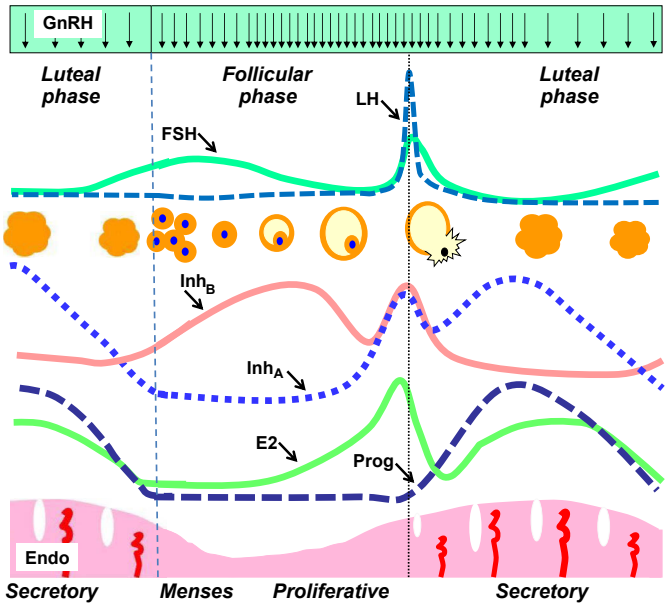

FIGURE 37.1 The hormonal, follicular, and endometrial dynamics of the normal menstrual cycle from the late luteal phase through menses and the beginning of a new cycle of follicle development, ovulation, and corpus luteum function and decline. Dynamic changes in the frequency of pulsatile gonadotropin-releasing hormone (GnRH) stimulate the integrated actions of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are responsible for: (1) follicular development with secretion of estradiol (E2), inhibin B (Inh_B), and inhibin A (Inh_A); (2) the preovulatory LH surge and ovulation; and (3) secretion of progesterone (Prog), E2, and Inh_A from the corpus luteum. Secretion of E2 and Prog result in proliferative and secretory changes in the endometrium (Endo), preparing it for implantation should conception occur. If conception does not occur, endometrial shedding follows the decline in hormone secretion, which accompanies demise of the corpus luteum. *From Hall JE.*[19]

quantity of GnRH released, while both gonadal steroids and the inhibins influence the pituitary response to GnRH. Progesterone has a profound inhibitory effect on GnRH pulse frequency,[20,21] which ultimately influences the differential control of LH and FSH synthesis; slow frequencies of GnRH stimulation of the gonadotrope, as seen in the luteal phase, favor the synthesis of FSH over LH.[22] Estradiol is an important negative feedback modulator of overall GnRH secretion,[23] and low levels of estradiol have a further effect at the pituitary level, suppressing LH and FSH responses to GnRH.[24] Finally, inhibin B and inhibin A selectively suppress FSH, acting at the pituitary alone (for review, see Welt[25]).

During the luteal-follicular transition in women (Figure 37.1), there is a preferential rise in FSH over LH. This is due, at least in part, to the loss of pituitary inhibition of FSH secretion by estradiol and probably inhibin A from the waning corpus luteum.[26] Increased GnRH secretion, made possible by the loss of progesterone and estradiol feedback, is also necessary during

the luteal-follicular transition to achieve levels of FSH that are adequate for follicular recruitment.[27] Thus, our working model is that slower frequency GnRH stimulation in the luteal phase facilitates pituitary synthesis of FSH whose secretion is inhibited by estradiol and inhibin A. With the declining levels of estradiol and inhibin A that accompany the functional demise of the corpus luteum in the late luteal phase, FSH secretion is released from negative feedback but requires restoration of more robust stimulation by GnRH to attain levels that are capable of recruiting a new cohort of follicles.

While increasing levels of FSH are necessary for recruitment of a new cohort of follicles at the beginning of each reproductive cycle, restraint of FSH is equally important to ensure that only a single follicle reaches the preovulatory stage in women. It is widely appreciated that estradiol secretion from developing follicles contributes to the negative feedback control of FSH as the follicular phase progresses (Chapter 28). Distinguishing an additional role for inhibin in this critical process in women has been challenging in the absence of the ability to administer inhibin or inhibin antagonists. Several lines of evidence, however, do suggest that inhibin feedback plays an important role in the dynamics of FSH secretion across the normal menstrual cycle. As will be discussed below, lower levels of inhibin B are associated with higher FSH levels during the luteal-follicular transition and serve as a marker of reproductive aging in women. In this setting and in ovulatory women with premature ovarian insufficiency (POI) in whom inhibin B is also low, FSH levels remain higher than normal across the follicular phase despite estradiol levels that are equivalent to or higher than normal.[28,29] In addition, in women in whom POI is due to autoimmune oophoritis, FSH decreases in conjunction with follicle development and elevated inhibin B even in the virtual absence of estradiol.[30]

In women, generation of the preovulatory LH surge that is necessary for ovulation requires ongoing pulsatile GnRH secretion, but is otherwise fully dependent on the marked augmentation of gonadotropin responses to GnRH that results from the effect of rising levels of estradiol on pituitary gonadotropes[19] (see also Chapter 28). The mechanisms underlying the preovulatory surge in women stand in sharp contrast to those in other animal species in which increases in GnRH secretion, in many cases tightly entrained to circadian cues, augment the effect of positive estrogen feedback at the pituitary,[31] perhaps allowing for greater flexibility in responding to environmental signals. This difference between women and other mammalian species in the mechanisms underlying generation of the preovulatory LH surge becomes particularly important in providing a perspective from which to interpret and translate the data on reproductive aging in females across species.

Ovarian Aging in Women

In women, reproductive senescence is due, in major part, to depletion of follicles from the ovary. In the human ovary, the germ cell population reaches its maximum of 6–7 million at approximately 20 weeks of gestation when primordial follicles, consisting of primary oocytes surrounded by a single layer of flattened granulosa cells, begin to form. Primordial follicles, together with follicles that have grown to intermediate and small primary follicle stages, constitute the finite pool of resting follicles in the ovary (for review, see Gougeon et al.[32]). At the time of birth, the resting follicle pool has been reduced to approximately 1 million, primarily due to atresia, with a further reduction to 250,000 by the time of puberty. While the factors that control the early growth and development of follicles are incompletely understood, autocrine and/or paracrine actions of the transforming growth factor β (TGFβ) family appear to play a major role, as discussed in Chapter 21. Although FSH does not appear to be absolutely required for preantral follicular growth, it is possible that FSH may influence the early stages of folliculogenesis (see Chapters 28 and 29).

FSH increases during infancy, and this increase can last as long as 18 months in girls. During this time, estradiol levels are increased but are not high enough to indicate that a preovulatory follicle has formed, and ovulation does not occur.[33] In childhood, FSH is extremely low and does not rise again until the early stages of puberty, which can occur as early as 8 years old in normal girls. During and after puberty, follicles will leave the pool of resting follicles either by activation of further growth or by degeneration. The quiescent primordial follicles are recruited to further growth and differentiation through a highly regulated process that limits the size of each developing cohort. FSH provides a critical stimulus for recruitment of resting follicles into the growing follicle pool beginning in adolescence, while AMH, produced in granulosa cells from small growing follicles, restrains this effect of FSH.[34] It is from this growing pool of preantral follicles of 2–5 mm that the follicle destined to ovulate is selected. Further FSH-dependent development is supported by estradiol, activin, and other intraovarian factors.

The age at which ovarian function is ultimately lost is based on both the size of the initial primordial follicle pool and the rate of depletion of this pool. In women, there is a strong correlation between the size of the resting follicle pool and the number of growing follicles. However, this relationship is altered with aging. Until approximately 30 years of age in normal women, the loss of follicles from the resting pool is primarily due to atresia, while after

this age an increasing proportion is lost due to entrance into the growth phase.[35,36] Thus, as the overall number of follicles within the ovary declines, the percentage of growing follicles increases,[36,37] potentially representing an adaptation that prolongs reproductive cycles and estradiol secretion but hastens the onset of menopause. In contrast to rodents, in which the loss of follicles can be described by a simple exponential, follicle loss in women is accelerated from approximately age 35 onward, with cessation of reproductive cycles when the pool of resting follicles is reduced to between 100 and 1000.[38]

Age-related changes in oocyte quality parallel the decrease in follicle number. Fertilization and conception rates are lower in older reproductive-aged women, and there is an increased early pregnancy loss with increasing age. While it has been suggested that these results may be due to preferential selection of follicles with higher quality oocytes early in reproductive life[39], there is mounting evidence that the process of aging per se has a profound effect on the oocyte and follicular components that were established in early life. The finite pool of oocytes is therefore subjected to internal and environmental factors that may be detrimental to oocyte and follicle quality. For example, there is evidence for increased reactive oxygen species in the aging follicle,[40] a sign of oxidative stress and possibly mitochondrial dysfunction.[41] In addition, there is evidence of a decrease in mitochondrial number and function in oocytes from women with higher basal FSH and reduced numbers of oocytes retrieved in IVF cycles, both indicators of decreased ovarian reserve.[39] Oxidative stress plays a significant role in tissue aging, profoundly affecting cellular mechanisms. DNA double-stranded breaks (DSBs) accumulate with age and DNA repair mechanisms are poorly activated in the oocyte, leading to apoptosis and diminishing survival (see Chapters 1 and 2). It has been shown that shorter telomere length is related to poor oocyte performance in older reproductive-aged women.[42] Even in nondividing cells like oocytes, oxidative stress depletes telomeres, the important DNA sequences that cap chromosomal ends, prevent genomic instability, and are essential for normal chromosomal segregation. Thus, the findings of reduced telomere length in older women are consistent with evidence that meiotic spindle assembly is dysregulated in this same population,[43] a process that may underlie the increase in chromosomal errors such as nondisjunction that occur with reproductive aging in women.

The above mechanisms are relevant to important environmental factors associated with accelerated depletion of the follicle pool: chemotherapy, radiation, and smoking. Chemotherapy increases DSBs resulting in significant damage to the resting state oocyte,[44] while radiation has a similar effect, but on the dividing granulosa cells rather than the oocyte. Components of cigarette smoke bind the polycyclic aromatic hydrocarbon receptor on both oocytes and granulosa cells, activating proapoptic genes.[45] These findings are consistent with reduction in the age of menopause by approximately 2 years in smokers compared with nonsmokers.[46,47] Other factors shown in epidemiologic studies to shorten the time to menopause are previous pelvic surgeries[48,49] and low socioeconomic status.[50] While the data are not entirely consistent, long-term oral contraceptive use has generally been associated with a later age at natural menopsuase.[51,52]

One of the most powerful predictors of age at menopause in epidemiologic studies is family history. In fact, twin studies estimate that 44–85% of the variance in age at natural menopause is heritable,[53–55] and genome-wide association studies (GWAS) have identified at least 17 genes that are associated with the age at natural menopause in women. One of the initial genes implicated in age at menopause is that encoding mini-chromosome maintenance protein 8 (MCM8) on chromosome 20,[56] which is necessary for genome replication and is expressed in human oocytes.[57] Abnormalities in its function may therefore relate to crossover nondisjunction, a characteristic of aged oocytes. A meta-analysis of 22 GWAS in ~40,000 women with replication in ~14,500 women[58] confirmed previous identification of four loci associated with age at menopause on chromosomes 5, 6, 19, and 20[56] and identified 13 new loci. The 17 loci account for ~4% of the variability in age at menopause. Together, they function in diverse pathways including hormonal regulation, DNA repair, and immune function. Biological pathway analysis was consistent in emphasizing general biologic pathways for mitochondrial function, DNA repair, cell cycle and cell death, and immune response.[58] The importance of the DNA repair process was further emphasized by Titus et al. who demonstrated the obligate role of DSB repair genes, including *Brca1*, in oocyte survival in mice.[59] In addition, in young women with *BRCA1* mutations, reduced follicle reserve as reflected by low levels of AMH was observed.[59] Finally, it has recently been shown that specific genotypes confer an increased risk of earlier menopause in women who smoke that is dependent on the degree of smoking, is considerably higher than the increase due to smoking alone, and is not seen in women who do not carry these genotypes.[60] Together, these observations suggest that such gene–environment interactions are likely to contribute to the wide range in age at menopause in normal women.

Aging of the Hypothalamic-Pituitary Axis in Women

Response to the Loss of Ovarian Feedback

With the decline and eventual cessation of ovarian function, the hypothalamus and pituitary are freed from the negative feedback of ovarian steroids and peptides. There is a dramatic rise in serum levels of LH and

FSH due to both an increase in GnRH secretion and an increase in the pituitary response to GnRH as discussed below. The loss of ovarian steroid feedback accompanied by the loss of inhibin is associated with a 15-fold increase in FSH after menopause compared to a 10-fold increase in LH after menopause.[61]

THE HYPOTHALAMUS

Measurement of GnRH levels in peripheral blood does not accurately reflect hypothalamic GnRH secretion, and thus, indirect methods must be used to gain insight into hypothalamic function in women. Comparative data from animal models demonstrating that LH pulses are directly linked to antecedent pulses of GnRH[62] form the basis for the traditional use of the frequency of pulsatile LH secretion as a surrogate for GnRH pulse frequency. These data are supported in men and women by the recreation of LH pulses by exogenous GnRH administration in patients with GnRH deficiency[63] and by the abolition of LH pulses by high-dose GnRH antagonist administration in normal women.[64] While the glycoprotein-free alpha subunit (FAS) is secreted from the gonadotrope and the thyrotrope in response to GnRH and TRH, respectively, studies in euthyroid GnRH-deficient patients receiving GnRH and in normal euthyroid subjects following high-dose GnRH antagonist administration demonstrate that pulses of FAS reflect pulsatile secretion of GnRH, while tonic secretion of FAS is supported by TRH.[63,64] In settings of fast frequency GnRH secretion, as would be expected in postmenopausal women, FAS is a superior marker of pulsatile GnRH secretion due to its shorter half-life.[63] In addition, while the clearance of both LH and FSH are prolonged in postmenopausal women in association with estrogen deprivation,[65,66] the FAS clearance is not similarly affected.[65] Using FAS as a marker of GnRH secretion and a blood sampling interval of every 5 min to avoid underestimating the underlying GnRH pulse frequency, it has now been shown that secretory pulses occur at an interval of approximately every 50 min in postmenopausal women. This compares to significantly longer interpulse intervals of every 90 min in the early follicular phase and every 90 min to every 4 h across the luteal phase,[67] but is not significantly different from 55 min intervals in late follicular phase and during the midcycle surge (also determined using FAS and a 5 min sampling interval[68]).

Pulse frequency reflects only one aspect of GnRH secretion and cannot be assumed to reflect the overall quantity of GnRH secreted as the amount of GnRH secreted per bolus may also vary. Peptidergic GnRH antagonists that compete with GnRH for binding to the GnRH receptor[69] can be used to estimate the amount of endogenous GnRH being secreted. In this way, the change in LH as a function of GnRH antagonist dose is first used to define the dose–response relationship and a submaximal GnRH antagonist dose that allows competition at the GnRH receptor is then selected. The change in LH in response to incomplete GnRH receptor blockade, expressed in relation to pre-antagonist LH levels to control for variations in GnRH receptor number or postreceptor effects, can then be used to estimate the overall amount of endogenous GnRH being secreted.[70] Thus, at a given submaximal GnRH antagonist dose, the percent change in LH is inversely proportional to the amount of endogenous GnRH with which the antagonist is competing. Equivalent doses of a GnRH antagonist were less effective in suppressing LH secretion in postmenopausal compared with premenopausal women, indicating that the overall quantity of GnRH being secreted in postmenopausal women is greater than in premenopausal women.[23]

GnRH expression in the medial basal hypothalamus (MBH) is higher in postmenopausal compared with premenopausal women studied at autopsy,[71] consistent with the above findings in healthy young postmenopausal women. Menopause is accompanied by hypertrophy of a separate group of neurons in the infundibular (arcuate) nucleus that express kisspeptin (KiSS-1), neurokinin B (NKB), substance P, dynorphin, and estrogen receptor alpha (ERα) gene transcripts, but not GnRH.[72] KiSS-1, NKB, and ERα expression in these neurons is higher in postmenopausal compared with premenopausal women, while the number of these neurons expressing dynorphin is lower in postmenopausal women.[72] Studies in a number of animal species provide significant support for communication between neurons that colocalize KiSS-1, NKB, DYN, and ERα with GnRH neurons, suggesting that this may be a mechanism by which estradiol exerts negative feedback on GnRH and gonadotropin secretion, as discussed in Chapters 11, 26–28, and 33. The autopsy studies in women discussed above provide support for a similar mechanism in postmenopausal women, with increased NKB and KiSS-1, which are stimulatory to GnRH, but decreased DYN, which appears to be inhibitory.

STEROID NEGATIVE FEEDBACK The study of postmenopausal women provides the opportunity to examine the effect of estrogen feedback on gonadotropin secretion through administration of exogenous steroids and to determine whether feedback occurs at the hypothalamus, pituitary, or both. In this population, a month of estrogen administration, resulting in early to mid-follicular phase estradiol levels is associated with a decrease in mean levels of LH, FSH, and FAS,[73] although not to premenopausal levels. FAS pulse frequency does not change with this dose and duration of estrogen administration.[73] However, the overall amount of GnRH, assessed using submaximal GnRH receptor blockade as described above, decreases in response to a

similar regimen of estradiol replacement.[23] Furthermore, positron emission tomography (PET) studies using flurodeoxyglucose (FDG) uptake provide evidence for a decrease in metabolic activity in the medial basal hypothalamus after only 24 h of low-dose estradiol administration in postmenopausal women.[74] Taken together, these studies provide robust support for a hypothalamic site of estrogen negative feedback in women. In postmenopausal women, addition of 7 days of progesterone to achieve mid-luteal phase levels to low-dose estradiol resulted in further decreases in LH, FSH, and FAS.[73] FAS pulse frequency was markedly reduced[73] in conjunction with a reduction in the overall amount of GnRH[23] beyond that induced by estradiol alone, consistent with a hypothalamic site of progesterone negative feedback in women.

THE PITUITARY

While it is now well known from animal and human models that low-dose estrogen plays a key role in defining the gonadotropin secretory profile by inhibiting hypothalamic secretion of GnRH, the presence of a direct pituitary site of estrogen negative feedback has been more difficult to establish, particularly in women with an intact hypothalamic-pituitary axis. To overcome this limitation, the pituitary was isolated from endogenous GnRH input in postmenopausal women using a GnRH antagonist dose that completely inhibits pulsatile LH secretion by blocking the LH response to endogenous pulses of GnRH.[24] Because the GnRH antagonist is a competitive GnRH receptor blocker,[69] it was then possible to administer a range of doses of exogenous GnRH that could overcome the degree of blockade required to inhibit the LH response to endogenous GnRH, and to produce LH pulses that spanned the range of LH pulse amplitudes in postmenopausal women. This model thus served as a hypophysiotropic clamp, permitting control of the dose and interpulse interval of pituitary exposure to GnRH, both of which influence the LH response to GnRH. Results of these studies demonstrated a direct pituitary site of estrogen negative feedback on LH and FSH in women and a greater pituitary effect of negative feedback on FSH than LH,[24] contributing to the differential control of FSH and LH secretion. This view is consistent with studies of GnRH-deficient women receiving invariant pulsatile GnRH replacement regimens (see Chapter 28).

Extremely low to unmeasurable levels of LH and FSH in GnRH-deficient men and women, and their increase with GnRH replacement,[67] indicate that GnRH is absolutely required for normal secretion of both gonadotropins. However, administration of a GnRH-antagonist dose that results in a decrease in LH of over 90% suppresses FSH by only 40%,[75] consistent with dual control of FSH secretion in the setting of an intact HPG axis and the known selective stimulation of FSH by the autocrine and paracrine effects of activin in the pituitary.[25] Loss of estrogen negative feedback at the pituitary, which is greater for FSH than LH, combined with loss of the negative feedback of inhibin on activin-stimulated FSH, may contribute to the greater rise in FSH than LH at menopause.

Hypothalamic-Pituitary Aging and Changes in Steroid Feedback

As will be discussed below, there is significant evidence that age-related changes in the hypothalamus and pituitary play a critical role in reproductive aging in rodents, and it has been hypothesized that similar changes may contribute to reproductive aging in women.[76,77] During the menopause transition in women, however, the decline in ovarian function is associated with dramatic fluctuations in ovarian feedback on the central components of the reproductive axis, making it difficult to determine the potential contribution of aging of the hypothalamus and/or pituitary per se to reproductive senescence. The complete cessation of ovarian function, however, creates an open-loop setting, in which the hypothalamic and pituitary components of gonadotropin secretion can be examined as a function of aging. It has now been shown that there is a 30–40% decrease in both LH and FSH levels after menopause between the ages of 50 and 75,[61,78] providing evidence that aging itself influences the hypothalamic and/or pituitary components of the reproductive axis; although even in women in their eighth decade, FSH and LH are markedly elevated compared with follicular phase levels of these hormones in reproductive-aged women.

THE HYPOTHALAMUS

Using FAS as a marker of underlying GnRH secretion, as described above, and a 5 min sampling interval to maximize the chance of detecting the gonadotrope response to fast-frequency GnRH pulses, it has now been shown that GnRH pulse frequency decreases with aging after menopause from approximately one every 50 min in younger postmenopausal women to approximately one every 70 min in their older counterparts.[79] These studies resolve prior discrepancies in the literature based on the use of LH as a marker of GnRH secretion and a 10 min blood sampling interval.[78,80,81] In contrast, aging in postmenopausal women is associated with a further 14% increase in the overall amount of GnRH secreted[23] from that already due to the loss of ovarian function described above. Taken together, these studies suggest that there is an increase in the pulse amplitude of GnRH secreted with aging, although this does not completely compensate for the 22% decrease in GnRH pulse frequency with aging. These complex hypothalamic changes do not appear to completely account for the 30% decrease in LH and FSH

with aging after menopause in women, suggesting that aging may also dampen the pituitary response to GnRH.

THE PITUITARY

Changes in the ability of the pituitary to respond to GnRH as a function of aging have been examined using LH pulse amplitude[79,80,82,83] or the LH response to pharmacologic doses of GnRH,[80,83] with inconsistent results. Some of this variability may be due to difficulty in accurately identifying endogenous LH pulses in postmenopausal women. In addition, the assessment of the pituitary response to GnRH as a function of aging may be confounded by the decrease in GnRH frequency with aging and the known increase in gonadotropin response to GnRH with longer interpulse intervals.[84] Using the hypophysiotropic clamp model, described above, in which it is possible to control both the amount and prior interpulse interval of GnRH simulation of the pituitary, studies have now demonstrated that the pituitary response to GnRH is significantly attenuated with aging; both LH and FSH responses were 30% lower in older compared to younger postmenopausal women.[85]

STEROID NEGATIVE FEEDBACK WITH AGING

Studies of the hypothalamic and pituitary sites of steroid negative feedback and the effect of aging on both of these mechanisms have been performed in postmenopausal women. Estrogen negative feedback at the hypothalamic level remains intact in older compared with younger postmenopausal women. Thus, a month of low-dose estrogen administration is associated with a significant decline in circulating levels of LH, FSH, and FAS, and a parallel decrease in the overall amount of GnRH, estimated using submaximal GnRH blockade, with no effect on FAS pulse frequency in either age group.[23,73] Similarly, addition of progesterone resulted in a decrease in FAS pulse frequency in older and younger postmenopausal women accompanied by the expected decrease in overall amount of GnRH that did not differ with aging.[23,73] At the pituitary level, the direct effect of estrogen negative feedback on FSH is attenuated with aging, while the effect on LH is unchanged.[24]

ESTROGEN POSITIVE FEEDBACK WITH AGING Several lines of evidence have suggested that sensitivity to estrogen positive feedback may be lost with aging in women.[86,87] In women who collected daily urine samples for variable periods of time during the menopause transition, failure of positive feedback was observed in a small percentage of women despite similar peak late follicular phase estradiol levels, leading the authors to conclude that positive feedback was impaired in these women.[76] However, the overall pattern of estrogen exposure was attenuated in those who did not ovulate compared to those who did, and it is well known that both the dose and duration of estrogen exposure are critical for generation of gonadotropin surges in women.[88,89] In studies using a controlled graded steroid infusion paradigm that recreated early and late follicular phase levels of estradiol in younger and older postmenopausal women, the LH surge generated in the face of identical preovulatory patterns of estradiol was attenuated with age.[85] In these same studies, FDG-PET showed a dramatic increase in metabolic activity at the pituitary, but not the hypothalamus, in association with the LH surge.[74] Given the importance of increased pituitary responsiveness to GnRH in generation of the LH surge in women, attenuation of the steroid-induced surge with aging is consistent with the studies discussed above that demonstrate a decrease in pituitary responsiveness with aging.[90]

Integration of the Reproductive Axis with Aging in Women

From a physiologic perspective, the process of reproductive aging can be divided into two primary mechanistic periods: (1) *early ovarian aging* when cycles remain regular in the setting of interrelated "compensatory" hormonal changes; and (2) the *menopause transition*, during which there is marked variability in the ovarian response to gonadotropin stimulation and in ovarian feedback on gonadotropin secretion before the complete loss of reproductive function.

EARLY OVARIAN AGING: COMPENSATORY CHANGES

The age-related decrease in the number of follicles in the ovary is reflected in the number of antral follicles seen on ultrasound. Antral follicle count (AFC) is decreased as a function of age,[37,91,92] and declining AFC is associated with decreasing fertility.[93] AMH, which is expressed in small growing follicles,[34] reflects the declining pool of follicles in the ovary[37] and is relatively stable across the menstrual cycle.[94] AMH is strongly and directly associated with AFC, and both have been used extensively as predictors of outcome in in vitro fertilization (IVF) cycles.[93] Inhibin B is constitutively secreted from granulosa cells of growing follicles and increases in response to FSH[95] as a reflection of the dramatic increase in granulosa cell number that accompanies follicle recruitment. While inhibin B measured on day 3 of the menstrual cycle is also inversely related to age and the size of the follicle pool, it is less useful as a marker of ovarian aging and fertility potential because of its variability in relation to FSH dynamics across the cycle.

Inhibin B, however, is a particularly important gonadal feedback modulator of FSH secretion in the period of early ovarian aging. The selective increase in FSH over LH (Figure 37.2) that reflects the reduction in ovarian function and fertility in women ≥35 is associated with lower levels of inhibin B.[7,28] This increase in FSH

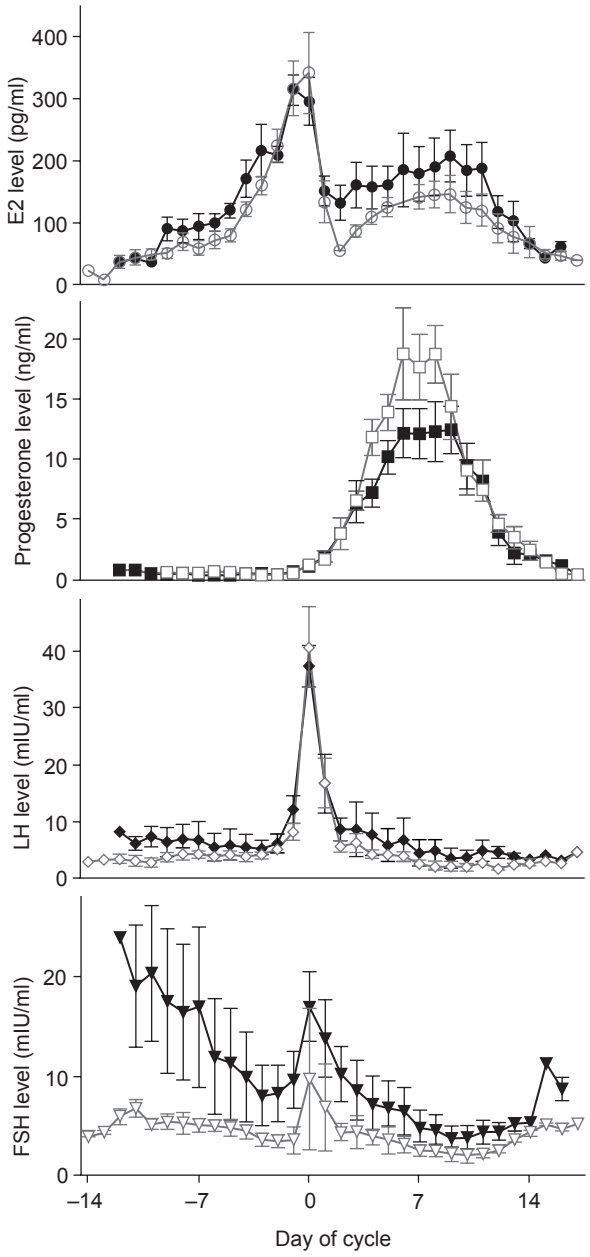

FIGURE 37.2 Relationships between estradiol (E2), progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) over the course of a normal menstrual cycle in midreproductive women aged 18–38 (open symbols) and older reproductive women aged 43–53 (closed symbols). Hormones are standardized to the day of the LH surge (cycle day 0). Note that FSH concentrations in older women are elevated in the presence of comparable (or slightly elevated) levels of estradiol. Inhibin B levels (not shown in this figure) were lower during the follicular phase in older women. *Reprinted with permission from Santoro N et al.*[28]

drives increased recruitment of follicles into the growing pool and is responsible for the increased rate of spontaneous dizygotic twinning in older women[96] and the increased follicular phase estradiol levels in the face of the declining follicle pool in early reproductive aging even in the absence of development of more than one

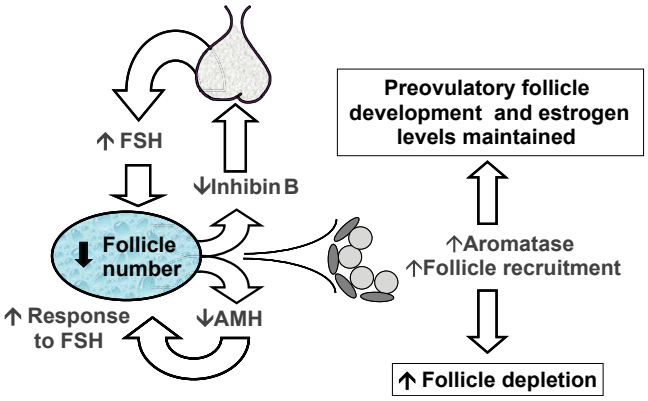

FIGURE 37.3 In early reproductive aging, the decrease in ovarian follicle number results in a decrease in inhibin B feedback on follicle-stimulating hormone (FSH) secretion from the pituitary and increased FSH levels. The decrease in follicle number is also associated with lower levels of anti-Mullerian hormone (AMH), increasing the ovarian response to FSH with: (1) increased recruitment of follicles into the growing pool from which the dominant follicle will be chosen and accelerated follicle depletion; and (2) an increase in aromatase activity, which also helps to maintain estradiol levels.

preovulatory follicle.[97] AMH restrains the stimulatory actions of FSH on recruitment of primordial follicles into the growing pool[34] as well as the FSH-dependent stimulation of aromatase,[98] but declines during this period due to the loss of follicles. A decrease in the androstenedione-to-estrone ratio in the follicular phase has been demonstrated in regularly cycling women ≥35 compared with their younger counterparts, consistent with increased aromatase activity.[99] It thus appears that lower levels of AMH in conjunction with elevated levels of FSH drive the accelerated depletion of the resting follicle pool after age 35 and are important for maintaining estradiol levels in the face of other evidence of declining ovarian function (Figure 37.3). Taken together, these integrated changes serve to extend fertility and maintain reproductive cycles and estrogen levels in the early stages of reproductive aging.

THE MENOPAUSE TRANSITION: MARKED BY VARIABILITY

The onset of irregular cycles marks the transition from the late reproductive years to the menopause transition. Large-scale cohorts have provided important insights into the overall pattern of hormonal changes that occur through this final transition to the end of reproductive life.[100,101] Taken together these studies indicate that the reciprocal relationship between declining levels of inhibin B and elevated levels of FSH occur relatively early in the process of ovarian aging, as discussed above, while estradiol secretion is relatively preserved. In fact, estradiol levels may not reach their nadir until a year after the final

menstrual period due to intermittent follicular activity that is insufficient to result in ovulation or menses. The increase in FSH precedes that of LH during the transition to the final menstrual period because of the earlier loss of inhibin with its selective inhibition of FSH. In addition, even with complete loss of ovarian function at the time of the final menstrual period and beyond, FSH levels are consistently higher than LH, possibly reflecting the dual control of FSH negative feedback by gonadal steroids and inhibin. While testosterone levels are lower in postmenopausal compared with premenopausal women, serum levels decrease before the final menstrual period and correlate better with age than with menopausal status.[101]

These aggregate changes do not reflect the marked variability in hormone levels that occur between women and within an individual woman from month to month over the 2–5 years of the menopause transition. Data from studies in which urinary hormone levels were collected over months indicate that, along with ovulatory cycles, estrogen levels can be low for prolonged periods followed by variable periods of time in which estrogen levels are very high[102] (Figure 37.4). The inconsistent estrogen response to FSH is not well understood. However, there is evidence from Doppler studies of reduced ovarian blood supply in the aging ovary.[103,104] This finding is supported by higher levels of vascular endothelial growth factor (VEGF), a marker of tissue hypoxia, in older reproductive-aged women.[105] These data suggest that FSH stimulation of viable follicles may well be impaired by a mismatch between the progressively decreasing and scattered follicles and their blood supply in the aging ovary.

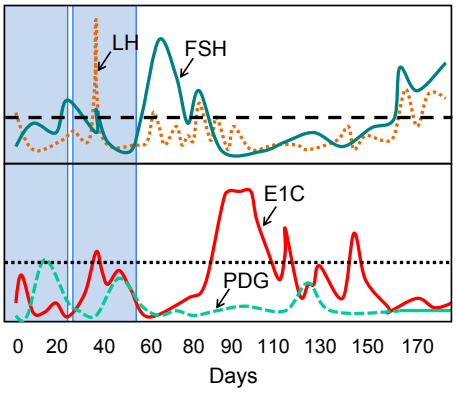

FIGURE 37.4 Daily urine samples over 6 months in a perimenopausal woman indicate marked variability in the pattern of LH, FSH, estrone conjugates (E1C), and progesterone diglucuronide (PDG). The dashed line in the upper panel indicates the upper limit of normal for FSH in young women, while the dotted line in the lower panel indicates the upper limit of normal E1C in young women. Shaded bars indicate cycles in which levels of PDG are consistent with ovulatory cycles. Adapted from Santoro et al.[102]

FSH levels are dramatically increased when estrogen levels are low, not only because of the loss of steroid and peptide feedback at the pituitary (Figure 37.5) but also because estrogen deprivation is associated with an increase in sialylation of FSH and a marked prolongation of its clearance.[66] Slowing of GnRH pulse frequency would also favor synthesis and secretion of FSH over LH. In the setting of variable responsivity to FSH, consistently elevated levels of FSH may subsequently drive follicle development and aromatase activity, accounting for estrogen levels during the menopause transition that may be two- to three-fold higher than in the preovulatory period of cycles in young women.[102]

During the menopausal transition, some increases in estradiol are followed by an LH surge, while some

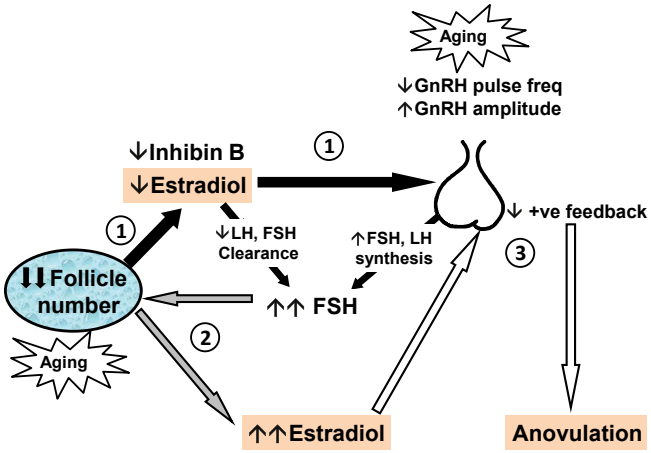

FIGURE 37.5 The transition to menopause is characterized by marked variability in the ability to recruit growing follicles from the aging ovary. Combined with the potential independent effects of aging on hypothalamus and pituitary, this variability results in three distinct patterns of hormonal changes and clinical symptoms: (1) the inability of follicle-stimulating hormone (FSH) to stimulate follicle recruitment results in low levels of both estradiol and inhibin B. These primary ovarian changes may be associated with vasomotor symptoms and sleep disturbance and result in the loss of gonadal hormone and peptide feedback at the pituitary. Combined with the possible age-related changes in hypothalamic gonadotropin-releasing hormone (GnRH) secretion (decreased pulse frequency and increased GnRH pulse amplitude), decreased negative feedback results in increased FSH secretion that is augmented by prolongation of the half-life of FSH secondary to low estradiol levels. The black arrows represent mechanistic changes that result in markedly elevated FSH levels; (2) markedly elevated FSH levels stimulate the remaining follicles, often resulting in extremely high levels of estradiol (gray arrows). Prolonged elevations in estradiol are frequently associated with marked breast tenderness, endometrial proliferation, and dysfunctional bleeding; (3) despite follicle development, abnormal patterns of estradiol, combined with the age-related loss of pituitary sensitivity to GnRH, results in inadequate LH surges and anovulation, further increasing the risk of irregular and heavy vaginal bleeding. These events repeat at irregular intervals before the complete loss of ovarian function following the final menstrual period and persistent hypoestrogenism.

are not. Generation of a preovulatory surge requires a highly specific pattern of increasing estrogen levels over an adequate duration,[88,89] both of which are likely to be altered in the face of asynchronous FSH and ovarian function as suggested by the pattern of estrogen excretion in anovulatory women transitioning to menopause.[76] The fact that the pituitary response to GnRH is attenuated with aging[90] (Figure 37.5) may also contribute to the approximately two-thirds of cycles in the year before the final menstrual period that are either anovulatory or have prolonged follicular phases,[106,107] resulting in irregular bleeding patterns along with varying breast tenderness, hot flashes, sleep disturbance, and possible mood changes.

UTERINE AGING

The ability to achieve and maintain a pregnancy in the setting of reproductive aging will be dependent on the uterus in addition to the hypothalamic, pituitary, and ovarian effects that have been discussed above.[108] Reports of successful pregnancies in women as old as 70 who received donor oocytes and exogenous hormonal support prior to implantation indicate that the aging uterus can support pregnancy. Early studies from donor oocyte programs that documented similar pregnancy, miscarriage, and delivery rates across all recipient ages provided support for this conclusion.[109–111]

However, more recent studies have documented an increased risk of preterm birth and low birth weight in donor egg recipients.[112] Other studies suggest that increasing age is associated with adverse outcomes that may be related to age-related myometrial dysfunction at the time of delivery. For example, increasing age has been associated with increasing stillbirth, after controlling for known maternal and fetal confounders.[113–115] In addition, increasing age is also associated with evidence of impaired contractile activity as there is an increased risk of cesarean section as well as longer duration of labor and operative vaginal delivery, when controlled for maternal and fetal confounders.[116] A biological basis for these associations is suggested by in vitro studies of uterine strips from mothers undergoing planned cesarean section that demonstrated that increasing age was also associated with reduced activity and increased multiphasic spontaneous myometrial contractions,[116] the latter of which have been associated with prolonged labor and an increased risk of emergency cesarean delivery.[117]

Standardization of Perimenopause/Menopause Terminology: STRAW+10

In 2001, the Stages of Reproductive Aging Workshop proposed a nomenclature and staging system for reproductive aging that included menstrual characteristics and qualitative hormonal changes.[9] The STRAW staging system facilitated research on the process of reproductive aging and the menopausal transition by introducing standardized terminology referenced to the final menstrual period. A decade later, our understanding of reproductive aging has advanced significantly. The studies of Hansen et al. confirmed the relationship of the decline in ovarian primordial follicle number from the peak reproductive to the late menopausal transition stages (STRAW stages −4 to −1) as defined by menstrual cycle characteristics.[118] FSH increased across the STRAW stages while AFC decreased. AMH also decreased across stages, but the ability of AMH to discriminate between stages was significantly compromised by assay sensitivity. While inhibin B tended to decrease across STRAW stages, changes were not significant, nor were changes in estradiol.

STRAW+10 incorporates a more detailed understanding of the hormonal changes that accompany ovarian aging both before and after the final menstrual period using information from STRAW+10 and other studies (Figure 37.6).[10] FSH cutoff levels are now possible because of international standardization of this measure. AFC and AMH have been used primarily in infertility populations, particularly in the setting of IVF. This, in addition to poor standardization of both measures across centers and poor sensitivity of AMH, limited the inclusion of AFC and AMH to qualitative descriptors in STRAW+10.

While issues of assay sensitivity have not been adequately resolved, the trajectory of AMH levels has now been described in large cohorts of girls and women from birth to post menopause, indicating an increase in AMH from birth to age 15, after which AMH levels plateau for a decade and begin to decrease with age after 25 years.[119] Application of AMH levels to longitudinal studies in normal women has now provided evidence that the combination of AMH and age,[120] and particularly the trajectory of AMH over time,[121] increases the precision of estimates of time to menopause in women over 35.

Hypothalamic-Pituitary Aging in Animal Models

Studies in rodents, primates, and other species permit a detailed understanding of structural and functional changes to the hypothalamus and pituitary with aging, while the use of experimental manipulations makes it possible to address the cause-and-effect of neural and hormonal changes and reproductive functional loss. There are a number of caveats to this approach, as the timing and progression of reproductive senescence in nonhuman species varies enormously,[122] with some animals maintaining reproductive competence to the very end of life and other species undergoing reproductive failure in mid-life, similar to women. In wild species,

Menarche FMP (0)

Stage	−5	−4	−3b	−3a	−2	−1	+1 a	+1b	+1c	+2
Terminology	Reproductive				Menopausal Transition		Postmenopause			
	Early	Peak	Late		Early	Late	Early			Late
					Perimenopause					
Duration	Variable				Variable	1–3 years	2 years (1+1)		3–6 years	Remaining lifespan
Principal Criteria										
Menstrual cycle	Variable to regular	Regular	Regular	Subtle changes in flow/ length	Variable length persistent ≥7- day difference in length of consecutive cycles	Interval of amenorrhea of ≥60 days				
Supportive Criteria										
Endocrine FSH AMH Inhibin B			Low Low	Variable* Low Low	↑ Variable* Low Low	↑ >25 IU/l** Low Low	↑ Variable Low Low		Stabilizes Very low Very low	
Antral Follicle Count			Low	Low	Low	**Low**	Very low		Very low	
Descriptive Characteristics										
Symptoms						Vasomotor symptoms *likely*	Vasomotor symptoms *most likely*			*Increasing* symptoms of urogenital atrophy

* Blood draw on cycle days 2–5 ↑ = elevated.
**Approximate expected level based on assays using current international pituitary standard.

FIGURE 37.6 Standardization of Menopausal Terminology: The Stages of Reproductive Aging Workshop +10 (STRAW+10) system for reproductive aging in women. FMP, final menstrual period; FSH, follicle-stimulating hormone; AMH, anti-Mullerian hormone. *From Harlow et al.*[10]

including humans prior to the last century, individuals rarely outlived the end of reproductive life.

While the hypothalamic control of reproduction is highly conserved in vertebrates in terms of the developmental organization, neuroanatomy, and functional importance of the GnRH system (see Chapter 33), menstrual cycles are limited to primates, meaning that a true menopause can only be studied in such species. Females of most other spontaneously ovulating species have estrous cycles that may differ substantially in length and in hormonal and physiological changes from these processes in women. This section will focus on experimental evidence of what is known about hypothalamic aging and regulation by steroid hormones during reproductive aging in female models. We will begin by discussing evidence in species in which most research has been conducted, laboratory rodents, and then compare and contrast nonhuman primate models. It should be noted that much research on release of GnRH has relied on measures of pulsatile LH and/or the LH surge. While a strong relationship between GnRH and LH often exists, the correspondence is not 100%, so caution is needed in attributing a change in LH levels to GnRH levels. We conclude with a brief overview of other aspects of pituitary and ovarian aging in animal models.

RODENTS

Rodents are an established laboratory model of reproductive aging, but rats and mice differ fundamentally from humans both in having a short estrous cycle (4–5 days) compared to the much longer menstrual cycle (~28 days) and in that their ovaries maintain viable follicles until relatively late in life.[123] Despite the presence of viable follicles, rodents undergo a transition from regular reproductive (estrous) cycles to irregular cycles to acyclicity at middle age,[124–126] indicating that the neuroendocrine system is driving this process independently of follicular loss.[123,127] Furthermore, transplantation studies of ovaries from aged to young ovariectomized rodents show that ovaries from old donors can begin to function in the young hosts, and undergo folliculogenesis and ovulation.[11] These findings suggest that reproductive failure with aging in rodents is not limited by the ovary. Therefore, rat and mouse models are useful in that they have enabled insight into how the hypothalamus changes with aging, both independent of and dependent upon gonadal steroid hormonal changes.

GnRH NEURONS

The hypophysiotropic GnRH neurosecretory system (referred to as GnRH1 neurons, but heretofore simply

called GnRH) of rodents comprises a population of about a thousand cells scattered in the preoptic area (POA) and basal hypothalamus[128,129] (see also Chapter 11). While sometimes fewer in number than in humans, and more rostral in distribution depending upon species,[1,130] the GnRH neuronal population in general is well conserved neuroanatomically in all mammalian species studied.[131] The GnRH system has been investigated for changes with aging, and results suggest little to no loss of GnRH perikarya numbers and no change in distribution.[132–138] Despite this, certain properties of GnRH neurons may change with aging. For example, the morphology, cytoarchitecture, and ultrastructure of GnRH perikarya and terminals change with aging,[139] suggesting that other properties of cells (e.g., their ability to synthesize or release the GnRH peptides) may occur.

Further evidence suggests a decrement in GnRH functional properties with aging. For example, experiments conducted in rats measuring pulsatile LH release, used as a proxy for pulsatile GnRH release,[140] show decreases in pulse amplitude in association with reduced mean concentrations of LH with aging. Similarly, the preovulatory LH surge is delayed and attenuated with aging.[141–145] GnRH mRNA, measured as an index of GnRH transcription, is elevated during preovulatory surges of young rats, but this is not detected in aging rats.[146,147] Finally, GnRH transcriptional activity, studied by co-expression of GnRH perikarya with the immediate early gene *Fos* or its gene product, is decreased in middle-aged compared to young rats during the preovulatory surge.[133,142] As a whole, these studies suggest a loss of activation of GnRH neurons in middle-aged rodents, prior to a loss of ovarian follicular complement. The fact that GnRH changes precede ovarian changes suggests at least some causality of the hypothalamus in reproductive senescence in rodents. However, because some of these studies focused on LH release as a proxy for GnRH release, it is not possible to discern hypothalamic from possible pituitary effects.

HYPOTHALAMIC NEURAL AND GLIAL NETWORKS THAT REGULATE GnRH

Hypophysiotropic GnRH neurons receive inputs from diverse stimulatory and inhibitory neurons, glia, and steroid hormone modulation (see Chapter 11). Therefore, aging of the hypothalamus is likely to include changes not just to GnRH cells and their properties but also to a broader hypothalamic network that controls GnRH synthesis and release. Of the many candidates for GnRH regulation during the entire life cycle, kisspeptin plays a dominant role, working in concert with other neuropeptides including neurokinin B and neuropeptide Y, neurotransmitters (glutamate, gamma-aminobutyric acid (GABA), norepinephrine) and neurotrophic factors (e.g., insulin-like growth factor 1 (IGF1), TGFα). Some neural inputs to GnRH neurons arise from projections to the GnRH cell bodies in the preoptic area, presumably to affect GnRH biosynthesis and/or to cause action potentials and subsequent release of the neuropeptide. GnRH dendrites also appear to be important for integrating inputs,[148,149] although this has not yet been reported in aging animals. Other inputs to GnRH neurons may occur locally at the site of GnRH nerve terminals in the median eminence. There, the stimulation of GnRH axons would result in increased GnRH neurosecretion, while inhibitory inputs to GnRH terminals would diminish GnRH release. It is important to differentiate between these three functional compartments of the GnRH cell because the source and nature of inputs to GnRH dendrites, cell bodies, and nerve terminals differs somewhat.[139,149,150] A discussion of evidence from rodent models showing structural and functional changes to selected systems, chosen for their key roles in GnRH regulation, and how their relationship to GnRH neurons changes with age follows.

KISSPEPTIN

In the rodent hypothalamus, there are two populations of kisspeptin neurons, one in the arcuate nucleus (ARC) and a second in the anteroventral periventricular nucleus (AVPV) in the anterior hypothalamus.[151] Hypothalamic kisspeptin signaling through its receptor, KISS1R, (aka GPR54 and encoded by the *KISS1R* gene) is critical for the acquisition of adult reproductive function[152] (see also Chapter 26), and in young adults, kisspeptin is crucial for preovulatory GnRH release.[153] Recent studies on hypothalamic kisspeptin show diminutions in its release and expression during the progression of reproductive decline, something that may impair GnRH gene activation and release. Gene expression of kisspeptin, and numbers of kisspeptin cells in the AVPV, are decreased during the LH surge of middle-aged compared to young rats.[154,155] This potential loss of drive to GnRH neurons is paralleled by the attenuated GnRH/LH surge described above. Replacement of kisspeptin via infusion into the preoptic area restored the attenuated LH surge in the middle-aged rats,[154] consistent with this idea. It is notable that in contrast to the AVPV, the population of kisspeptin neurons in the ARC does not undergo age-related change,[155] and this is important because it supports the hypothesis that the driving force to the GnRH/LH surge arises specifically from the AVPV and integrally involves kisspeptin inputs (see Chapter 11).

GLUTAMATE

Glutamate is considered to be the predominant excitatory neurotransmitter in the brain, and a well-known stimulatory regulator of GnRH cells.[156–158] Glutamate's actions on GnRH neurons occur through *N*-methyl-D-aspartate receptors (NMDAR)[159] and

non-NMDARs.[160,161] Glutamate activation of GnRH neurons is considered to be a critical event in the attainment of adult reproductive function,[158,162] and in adulthood, glutamate stimulates GnRH gene expression[163] and release.[164] With aging, there is decreased glutamate release in rodents[165] together with diminished response to its actions on postsynaptic membranes. Some of this age-related change is likely due to structural properties of glutamate receptors that confer functional responses to the ligand. Ionotropic glutamate receptors are made up of various combinations of subunits, the stoichiometry of which affect activation of the receptor and signal transduction.[166,167] Measurements of gene expression of NMDAR and non-NMDAR subunits demonstrate age-related changes in mRNA and protein in the hypothalamus[144,159,168] with likely functional consequences, as the ability of glutamate to activate GnRH neurons also diminishes with aging.[144,159,168] Protein expression of glutamate receptors,[122,159] and posttranslational regulatory processes (e.g., decreased phosphorylation of NMDAR subunits)[169] also occur in a manner that decreases the activity of glutamate. While initially controversial, it is now accepted that GnRH neurons co-express different classes of glutamate receptors,[137,163,170] and subpopulations of GnRH neurons respond to NMDA and non-NMDA pharmacological agents in electrophysiological studies.[171,172] Of relevance, the co-expression of GnRH neurons with glutamate receptor protein suggests stoichiometric changes in the ratio of receptor subunits, especially NR2a and NR2b, in aging compared to young female rats.[137] Thus, the stimulatory drive to GnRH neurons from glutamate decreases in the aging rodent hypothalamus. Interestingly, a pharmacological study on the interplay among glutamate, kisspeptin, and GnRH signaling revealed that glutamate and kisspeptin may modulate each other's activity on GnRH cells and contribute to the loss of GnRH output with aging.[154]

GABA

There are a few reports about another key amino acid neurotransmitter in the brain, GABA, and its regulation of the GnRH system during reproductive aging. In general, results suggest increased GABAergic signaling in the aging hypothalamus. Measurements of GABA release in the POA by microdialysis showed higher levels in middle-aged than young female rats.[173] Elevated gene expression of an enzyme involved in GABA biosynthesis, glutamic acid decarboxylase 67 (GAD67), was shown in middle-aged compared to young rats.[174] These results are consistent with pharmacological responses of GnRH neurons to GABAergic agonists/antagonists, although it is important to point out that electrophysiological responses of GnRH neurons to GABA are stimulatory, so they do not mirror the inhibitory pharmacological actions.[175]

STEROID HORMONE RECEPTORS AND FEEDBACK

A critical component to understanding age-related changes in the hypothalamic control of reproduction is how negative- and positive-feedback actions of ovarian steroid hormones undergo alterations with aging. While such changes have been reported, the mechanisms for and the consequences of such changes are still relatively unknown. The field of steroid hormone biology has expanded greatly in the last 20 years through discoveries of new nuclear and membrane receptors that bind estrogens and progestins (among other steroids, as described in Chapter 9). The nuclear estrogen receptors (ER) α and β and the progesterone receptor (PR) are abundantly expressed in the hypothalamus, with heterogeneous distribution across hypothalamic subnuclei.[176–178] Although GnRH neurons do not express ERα or PR (or if they do, it is at extremely low levels), they express ERβ, but this cannot explain most of the feedback actions of estradiol[179] (reviewed in Ref. 180; see also Chapter 11.). Several converging lines of research identified a membrane ER, GPR30 (also called GPER),[181,182] demonstrated the membrane localization of "nuclear" ERs,[183] identified other ER-responsive membrane ion channels,[184] and identified membrane progestin receptors (PGRMC).[185] These nonnuclear receptors are expressed in the brain, and their discovery has provided significant insights into some of the rapid actions of ovarian steroid hormones on the hypothalamus that cannot be explained by actions of nuclear hormone receptors acting as transcription factors. However, to our knowledge there are no studies on age-related changes in these membrane receptors, so future research is needed in this area. This is particularly important since there is emerging evidence for co-expression of GPER on GnRH neurons,[186] suggestive of a direct site of estradiol action on GnRH cells.

There are functional losses of both negative and positive feedback actions of estradiol and progesterone in the aging rodent hypothalamus. As discussed above, both endogenous LH surges in aging intact rats and steroid-induced LH surges in OVX rats are delayed and attenuated (reviewed in Ref. 187), indicative of a decrease in positive feedback. Furthermore, as rats transition from early persistent estrus (PE) to longer-term PE, steroid-induced positive feedback responses to estradiol plus progesterone declines.[188] Although in these studies peripheral LH was measured as a proxy for GnRH, these changes are consistent with the loss of activation of GnRH neurons as demonstrated by diminished FOS co-expression during the LH surge, in aging compared to young rats.[142,145] Similarly, negative feedback effects of steroids are also attenuated, as evidenced by the timing of the increase in LH in response to the removal of ovarian steroid feedback by OVX.[150]

Changes in steroid hormone gene expression have been reported in the aging brain in a few studies; these may relate to functional changes in steroid hormone feedback described above. For example, ERα mRNA levels in the periventricular POA are lower in old than middle-aged or young female rats.[189] Another study compared mRNA of ERα in POA and medial basal hypothalamus (MBH), showing no difference in POA but increased levels in MBH.[190] In addition, ERβ mRNA levels were higher in middle-aged than young rats in both of these regions.[190] PRs also undergo age-related changes, with decreased PR mRNA shown in AVPV of acyclic compared to cycling middle-aged female rats.[191]

Studies on protein expression of ERs and PR demonstrate changes, albeit relatively small ones, in the aging compared to the young rodent hypothalamus. No change in ERα cell numbers and density was found in aging, ovarian-intact rats.[192] Stereological measures of ERα cell numbers in four hypothalamic regions showed small but significant differences with age in AVPV and the ventromedial nucleus (VMN), with levels lowest in middle-aged compared to young and old rats (animals were OVX and given vehicle or estradiol treatment).[193] By contrast, no difference in ERα cell numbers in the medial POA and ARC was found in that same study. Measures of ERβ cell numbers in AVPV showed an age-related decline, but no change in the bed nucleus of the stria terminalis.[193] These results demonstrate the importance of regional specificity in performing these measures, as some but not all hypothalamic nuclei exhibited an age-associated change in steroid hormone receptor-immunoreactive cells. It is interesting that the AVPV is a site of dynamic changes in both ERα and ERβ cells because of this region's important role in the positive feedback signal from estradiol plus progesterone to induce the GnRH/LH surge, an absence of this event being a hallmark of reproductive aging in rodents.[141–145]

GLIAL-GnRH REGULATION

The hypothalamus expresses all of the major glial cells in the brain—astrocytes, microglia, and oligodendroglia—plus a modified astroglial cell, the tanycyte.[2] Tanycytes extend from the third ventricle to the median eminence, and GnRH fibers and terminals are interspersed among them.[194–196] Tanycytes contribute to the ability of secreted GnRH peptide to gain access to the portal capillary vasculature,[195] and dynamic changes in the relationship among tanycytes, GnRH terminals, and portal vessels occur in response to modifications in the steroid hormone milieu.[133,197,198] In young adults, tanycytes are organized in a largely linear fashion, with cell bodies near the third ventricles and processes extended to the pericapillary zone of the median eminence. This organization is lost with aging,[139,199] although the mechanism for this change and its functional significance is not well understood and to date has only been studied in OVX rats.

Further insight to the GnRH-glial connection with aging is provided by two studies. In one, direct contacts between GnRH membranes and tanycytes were quantified in the median eminence of young, middle-aged, and old OVX rats.[199] Results showed a small but significant age-related decline with aging. In another study, the association of GnRH neurons with astrocytes was determined in rostral POA, and shown to be both lower in middle-aged than young rats and to undergo fewer diurnal changes in the older group.[200]

Several other lines of research have indicated that the responses of GnRH neurons to glial neurotrophic factors declines with aging (reviewed in Ref. 194). For example, two members of the epidermal growth factor family, transforming growth factor-α (TGFα) and neuregulin 1β, indirectly stimulate GnRH release via signaling through the epidermal growth factor family of receptors (ERBB) that are expressed on hypothalamic glial cells.[195,201,202] During the GnRH/LH surge, there are temporal changes in expression of *Erbb4* (ERBB4 receptor) mRNA levels in the median eminence, ARC, and POA, and these are diminished in middle-aged compared to young rats.[203] Similar effects were seen for *Erbb1* mRNA.[204] Neuroanatomical work complimented this molecular work to show that immunolabeling of ERBB receptors differs between young and middle-aged rats.[203,204] As a whole, studies on GnRH-glial relationships suggest prominent age-related changes in structure and function, and a potential role in the attenuated GnRH/LH surge.

In summary (Figure 37.7), rodent models of reproductive aging demonstrate changes to functional and morphological properties of GnRH neurons. Neural and glial regulatory inputs to GnRH neurons appear to undergo a shift from being dominated by excitatory inputs to being dominated by inhibitory inputs. Changes in expression of receptors on GnRH neurons with age further contribute to a loss of responsiveness of GnRH cells to regulatory inputs. As a consequence, pulsatile GnRH release diminishes and there is attenuation of the GnRH/LH surge, with an overall decrease in the drive from GnRH neurons to the pituitary, and subsequently, the ovary.

NONHUMAN PRIMATES Studies on reproductive aging in nonhuman primates are limited by availability of aged monkeys, and work may be confounded by not knowing or being able to control the full life history of a 20- to 30-year-old animal. Nevertheless, the Old World macaques are an important model of aging because they undergo a natural menopause, beginning with irregularities in menstrual cycles and culminating in complete follicular atresia, albeit much later in their lifespan than in women.[205–207] New World monkey species may be more limited in that they do not experience

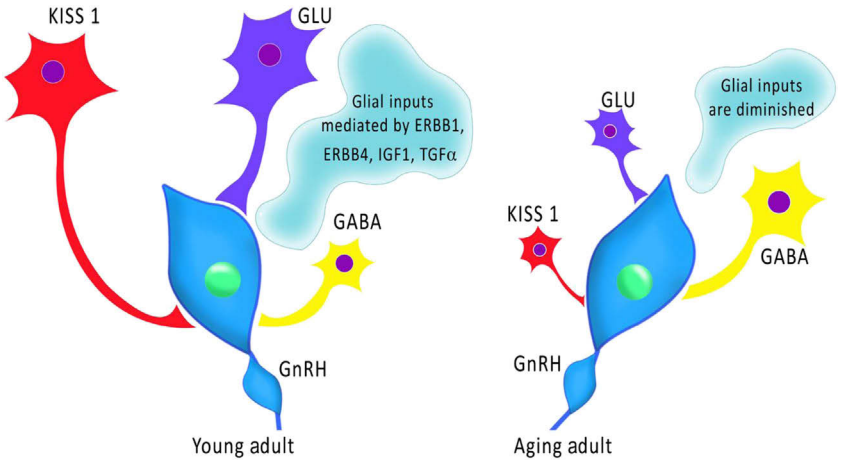

FIGURE 37.7 Schematic diagram showing age-related changes to hypothalamic regulators of gonadotropin-releasing hormone (GnRH) neurons, from the female rodent literature. In young adults (left), GnRH neurons receive excitatory inputs from kisspeptinergic (KiISS1) neurons from the anteroventral periventricular nucleus (AVPV). GnRH release is also strongly affected by excitatory glutamatergic (GLU) inputs, acting upon *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors. Pharmacological studies suggest that GABAergic inputs are relatively modest at that age. In addition, glial cells produce neurotrophic factors through ERBB1, ERBB4, insulin-like growth factor 1 (IGF1), and transforming growth factor α (TGFα). In aging adults (right), the balance of these neurotransmitters and neurotrophic factors shifts, with the relative size of inputs to GnRH neurons shown smaller or larger compared to the young adult. GABA, gamma-aminobutyric acid.

a complete loss of follicular reserves nor do they exhibit menstrual cycles.[208] Regardless, the monkey literature is very important because it fills gaps in information that are more directly relevant to aging women with menstrual cycles, and which cannot be obtained in another way in humans.

GnRH NEURONS

Pulsatile GnRH release was measured from nerve terminals by push–pull perfusion of the median eminence of young adult and perimenopausal aging adult female rhesus monkeys, with results showing significant increases in mean GnRH concentrations in perimenopausal females.[209] That study also demonstrated that some aged monkeys had exceptionally high GnRH pulses. These results are consistent with increased hypothalamic GnRH mRNA in postmenopausal compared to young OVX rhesus monkeys,[210] and consistent with findings that humans also have elevated GnRH mRNA with aging,[71] underscoring the translatability of the monkey work to women. Measurements of serum gonadotropins and steroids in the same study in which pulsatile GnRH release was measured[209] demonstrated that LH and FSH were higher in aged monkeys (FSH significantly so), estradiol was decreased in the aged animals, and there were no differences in progesterone. Another study measuring pulsatile LH release in perimenopausal female rhesus monkeys demonstrated significantly higher mean concentrations and pulse amplitude compared to young monkeys,[211] consistent with the GnRH findings.[209] Longitudinal daily measures of serum reproductive hormones over several months in pre-, peri- and postmenopausal rhesus monkeys added to this work by showing aging-related increases in FSH and decreases in estradiol.[205] While LH did not differ significantly in overall levels in that study, the pattern of release was different. Decreased serum progesterone was also observed in the postmenopausal group, presumably because these animals were not ovulating and thereby not generating a corpus luteum.[205] Inhibin B and anti-Mullerian hormone were also measured in this latter study, both of which decreased with aging. This decrease in inhibin may also contribute to the increase in FSH, without an overall change in LH, in the aging macaques. As a whole, these studies on daily LH and FSH, pulsatile LH, and pulsatile GnRH release are consistent with the likelihood that decreased serum estradiol concentrations, and a reduction in negative feedback at the level of hypothalamus (and possibly directly at the pituitary), contribute to or are responsible for the aging-related increase in these hypothalamic and pituitary hormones. It is noteworthy that these age-related increases in GnRH and gonadotropins are the opposite of those reported for rodents (discussed above) and suggest fundamental species differences in these senescent processes.

Changes in GnRH and gonadotropin release in aging nonhuman primates are not reflected by neuroanatomical changes to the GnRH population. A study that counted GnRH cell numbers in female monkeys from ages 2–21 years detected between 55 and 470 neurons per individual, with no difference in GnRH neuron numbers with age in monkeys.[132] Those numbers were likely an underestimate as only one side of the brain was counted, and the antibody penetrated through less

than one-third of the tissue thickness; therefore, results should be extrapolated by at least six-fold. Nevertheless, even extrapolated results suggest no age-related change in GnRH cell numbers, a finding that replicates rodent work discussed earlier.

KISSPEPTIN, NEUROKININ B (NKB), AND PRODYNORPHIN (PDYN)

The neuroanatomical regulation of GnRH neurons in primates differs somewhat from that in rodents. In mice and rats, there are two discrete populations of kisspeptin neurons, one in AVPV and one in ARC, postulated to regulate positive and negative feedback actions of gonadal steroids onto GnRH neurons, respectively. There is a broader distribution of kisspeptin neurons in sheep and monkeys, including in the ARC and POA, and their relative importance in mediating negative and positive feedback effects in females is less well understood.[212,213] Aged postmenopausal rhesus macaques have elevated *KISS1* and *KISS1R* gene expression compared to premenopausal (OVX) monkeys in MBH.[210] Eghlidi et al. also reported increased *KISS1* mRNA levels in perimenopausal compared to premenopausal rhesus monkeys.[214] Rance's group had previously used *in situ hybridization* in adult OVX cynomolgus monkeys and found that *NKB* gene expression was profoundly decreased by estradiol treatment.[215] Eghlidi et al.[214] further showed increased *NKB* mRNA, but no change in *PDYN* mRNA, in the ARC. When OVX monkeys were compared to intact or OVX + E2 aged monkeys, *KISS1* and *NKB* genes were highest in the OVX group (no E2) and lowest in the OVX + E2 (long term, 4 + years of E2 treatment).[214] By contrast, the gene expression of receptors for kisspeptin, neurokinin B, and prodynorphin, *KISS1R*, *NK3R*, and *KOR*, respectively, did not change with age in intact monkeys or with E2 treatment in OVX monkeys.[214] Taken together, these data on kisspeptin and neurokinin B suggest that these cells are sensitive to both age- and estradiol-regulated changes and that negative feedback is very important in determining levels of gene expression.

STEROID HORMONE ACTIONS AND RECEPTORS

ERα and ERβ are widely distributed in the monkey hypothalamus, and some age-related changes were reported,[216] demonstrating an age-related decline in ERα and little difference in ERβ. However, there is a dearth of studies on whether there is any loss of negative or positive feedback on steroid hormone receptor levels in the aging monkey hypothalamus. One report on ovarian-intact Japanese macaques analyzed aging-related changes in serum hormones, ovulation, and response to steroid feedback.[217] When peri- and postmenopausal monkeys were given injections of estradiol designed to induce an LH surge, the negative and positive feedback effects were preserved in the aging monkeys. This is an interesting result because it shows: (1) prior to injection of estradiol, the old monkeys had higher LH concentrations than the young monkeys; (2) injections of estradiol suppressed plasma LH levels at both ages to approximately a similar level, with the nadir reached at 0.5 days; and (3) a positive feedback surge of LH followed at both ages, with the peak at 2.5 days post injection. Although not discussed by the authors,[217] the old monkeys actually appear to be more sensitive than young monkeys to the estradiol, both in terms of the negative and positive feedback responses.

PITUITARY AGING IN FEMALES As discussed earlier, it is difficult to dissociate the hypothalamic-pituitary levels of the HPG axis when gonadotropin measurements in peripheral circulation are used as a functional readout of hypothalamic GnRH release. While the relationship between GnRH pulses and gonadotropin release, especially LH, often exhibits close to a one-to-one concordance,[218,219] this relationship may break down during aging, especially since pituitary responsiveness to GnRH may be affected by aging, as discussed in the next paragraph. Such a breakdown, if any, might relate to changes in GnRH pulse frequency and concomitant effects on pituitary gonadotropin synthesis or half-life. Furthermore, FSH changes with aging may reflect not only changes to GnRH regulation but also to aging-related changes in inhibin.[205] Therefore, LH is a much better proxy than FSH for reflecting GnRH dynamics in aging animals.

We will begin by briefly discussing evidence from animal models for age-related changes in pituitary responsiveness to GnRH. The literature is mixed, but most studies indicate that there is a diminution in this response with aging. For example, a study using incubated pituitaries from young and middle-aged female rats showed that while basal LH release was similar, GnRH-stimulated LH release was lower in the middle-aged group in an estrous cycle stage-dependent manner.[220] Another study found that both basal and GnRH-stimulated LH release was lower in pituitary cultures from middle-aged compared to young rats;[221] in the latter study, middle-aged rats were in PE, while in the former,[220] middle-aged rats were still cycling, possibly explaining differences in basal release. Further work found that middle-aged rats with attenuated LH surges have decreased pituitary GnRH responses in vitro, whereas animals whose surges have not yet attenuated have normal GnRH responses.[222] An in vivo study evaluating LH responses over time to three doses of GnRH found an age-related decrease.[223] These data in rodents are consistent with a loss of pituitary GnRH responsiveness in women[90] and ewes.[224] However, it should be noted that to the contrary, several studies show little or no change in pituitary LH responses to GnRH.[225]

Other properties of the anterior pituitary gland undergo age-related change in females, but there are surprisingly few studies in this arena. Quantification of receptor binding sites for GnRH in pituitary membranes of young, middle-aged, and aged female mice showed no change with aging.[226] Thus, any changes in LH responsiveness to GnRH with age is likely due to other properties of the gonadotropes, such as GnRH receptor sensitivity, rather than number of binding sites per se. Another group reported that pituitary LH content was lower in PE middle-aged compared to cycling young rats.[221] Consistent with this, LHβ gene expression decreased between young cycling and middle-aged PE rats.[227] Clearly, further research is needed in order to clarify changes that are intrinsic to the aging pituitary gland of females.

Ovarian Aging in Animal Models

Ovarian aging in women is not well modeled by most natural animal models of reproductive senescence. First, true menstrual cycles are limited to humans, Old World monkeys, and apes (reviewed in Ref. 228). While mammalian species with spontaneous ovulation (e.g., rodents, dogs, sheep, cows, etc.) have reproductive cycles, these species do not menstruate. Second, complete exhaustion of the follicular pool with aging is almost never detected in species other than higher primates and some whales. One reason for this is that shorter-lived species may not outlive their ovarian pool. However, even relatively long-lived species such as macaques do not experience a complete loss of follicles, if at all, until the last few years of their lives.[229] As an example, rhesus monkeys can live for 30 years but can be fertile until their late twenties, an age comparable to that of a woman in her 80s.[209] Apes such as chimpanzees and gorillas do have very few ovarian follicles at the end of life (reviewed in Ref. 208,217), but it is obviously not feasible to conduct extensive research in these species. The tamarin, a New World monkey, has been used to explore ovarian aging. While these animals do not have complete follicular exhaustion with age, animals undergo changes in cycle length and often become anovulatory with advanced age.[230] Numbers of ovarian and follicular cell types were quantified in young, middle-aged, and old individuals.[231] Primary follicles, small preantral follicles, and large preantral follicles decreased in number with age, with the greatest decrease seen for primary follicles. Tamarins also have a steroidogenic interstitial gland in the ovary that enables steroidogenesis, mainly of progestins and androgens, until very late in life.[208] Thus, the removal of steroid negative feedback that happens in women is not well modeled by this species.

Rat and mouse models of ovarian aging must be used with caution because of the maintenance of viable follicles through the lifespan (reviewed in Ref. 123). Mouse models may be slightly better than rats, as mice have earlier follicular loss relative to rats,[232] albeit probably not complete. Other models such as the 4-vinylcyclohexene diepoxide (VCD)-treated rat are becoming popular because VCD destroys small preantral follicles.[233] Again, though, this is an artificial manipulation that is valuable for neuroendocrine and other endpoints but does not provide information on natural mechanisms of ovarian aging. Finally, genetic models may be informative in this regard. The follitropin receptor (FORKO) heterozygous mouse, lacking the FSH receptor, undergoes reproductive senescence at about 7–9 months and has a loss of follicles with aging.[234]

Summary of Animal Models of Reproductive Senescence in Females

Rodent models of reproductive senescence are important because they enable investigators to differentiate between the contribution of the different levels of the hypothalamic-pituitary-ovarian axis to aging processes. In particular, studies in rats and mice have highlighted how hypothalamic neural networks involved in GnRH regulation play a primary role in a loss of reproductive capacity. Results of these studies indicate that GnRH neurons have the intrinsic ability to continue to synthesize and release the GnRH peptide with aging, but that other regulatory inputs to GnRH cells undergo substantial changes resulting in a loss of GnRH output to the pituitary gland. Neurons that synthesize kisspeptin and neurokinin B appear to be quite important in these age-related functional changes, and the balance between excitatory amino acids (glutamate) and inhibitory amino acids (GABA) are also involved. As discussed earlier, other neurotransmitters as well as neurotrophic factors, and expression of their receptors on GnRH neurons, change with aging. The net result of this neural reorganization is a loss of drive to GnRH neurons and, consequently, diminished output to the anterior pituitary.

Monkey models provide great translational relevance to understanding menopause, due to similarities in the menstrual cycles of Old World primates and women. Monkey work has shown the critical role played by estradiol negative feedback in young adults and the consequences of the loss of that feedback at menopause. While rodents also experience negative feedback regulation, hypothalamic responses in primates show some fundamental differences from those in rodents. For example, kisspeptin gene expression increases in aging women and monkeys, whereas it decreases in aging rodents. In primates the increased kisspeptinergic activity is attributable to the loss of negative feedback from ovarian estradiol. In rodents, in which estrogen levels are maintained with aging, the decreased kisspeptin

activity likely reflects a change in neural circuitry and function. Thus, each species provides novel information on hypothalamic and ovarian changes, and their interactions, in female reproductive aging.

MALE REPRODUCTIVE AGING

Gender Differences

Reproductive aging in men exhibits a number of striking differences from the female. The most fundamental difference is that unlike the fixed complement of follicles seen in women, the germinal epithelium of the male can continue to generate fresh gametes throughout life. Accordingly, the reproductive lifespan of men may be as much as several decades longer than their female counterparts. Second, while aging in the female is characterized by an inexorable decline in ovarian function with follicle exhaustion following a relatively predictable time course, the process in the male is more modest and gradual and shows a high degree of variability. Third, while gametogenesis and steroidogenesis are very tightly coupled in the ovary and are both susceptible to the impact of aging, the same is not true in the male in whom aging tends to have a greater impact on testosterone production than spermatogenesis.[235–237] For this reason, we will discuss these processes separately following the dual compartment model of the testis that involves FSH regulation of Sertoli cell function on the one hand and LH regulation of testosterone production by Leydig cells on the other (see Chapter 16).

Methodological Challenges

Although studies of reproductive aging in the male are not complicated by the cyclical changes in gonadal function associated with the ovarian cycle, a number of methodological challenges nonetheless exist. The ideal model in which to examine the pure impact of aging on the reproductive system would be one that enjoys relative disease-free longevity. In the human there are the challenges of distinguishing the impact of aging from age-related illness, the inability to access the hypophysial portal circulation to measure endogenous GnRH and the difficulty of procuring testicular tissue. While studies in nonhuman primates have been pivotal to our understanding of the hypothalamic-pituitary-testicular axis particularly with reference to the regulation of puberty,[238] relatively few studies have utilized this model to evaluate age effects. There are obvious challenges to evaluating aging in animals living in the wild, while the suitability of captive animals as a model for natural aging is debatable, and there are significant costs associated with housing these animals over long periods of time. The Brown Norway rat is a useful model for elucidating mechanisms underlying male reproductive aging as this strain enjoys relatively healthy longevity and like the human exhibits declining function both peripherally at the testis as well as centrally at the hypothalamus and pituitary.[239]

Reproductive Aging in Men

Spermatogenesis

While the association between advanced maternal age and declining fertility has been unequivocally established, the role of paternal age is more controversial.[237] The impact of aging on fertility and spermatogenesis in men has been comprehensively reviewed in the third edition of this textbook in an excellent chapter by Handelsman.[240] Studies of men younger than 35 years of age found no relationship between the age of the father and time to pregnancy.[241,242] However, studies with a greater span of paternal age demonstrate an age-related decline in fecundity.[243,244] For example, a large UK population study of 8515 planned pregnancies showed that after adjusting for other factors, the probability that an ultimately fertile couple will take >12 months to conceive nearly doubles from ~8% when the man is <25 years to ~15% when he is >35 years.[243]

The cause of this modest but consistent reduction in male fertility with age has not been established and could potentially reflect age-related reductions in sperm numbers and/or function, a reduction in the frequency of coitus, or paternally mediated defects in embryo viability. Efforts to understand the impact of age on semen analysis have been hampered by difficulty recruiting subjects from the general population, which has resulted in small sample sizes and selection bias, which, in turn, have limited the generalizability of the findings. Most studies of healthy older men have shown that aging is associated with modest reductions in semen volume, total sperm output, and sperm motility.[245–247] In one of the largest studies to evaluate the relationship between age and semen parameters, in 6022 Israeli men across seven age groups ranging from <25 to >55 years optimal semen parameters were observed in men aged 30–35 years, while the most significant abnormalities occurred in men over 55.[247] Semen volume started to decline at age 35 and was 37% lower in men over the age of 55 compared to those under 35. It is noteworthy that the decrease in semen volume with age occurred despite a concomitant increase in the duration of abstinence with age, which normally tends to increase ejaculate volume. Sperm motility also showed a significant change with age, decreasing from 44 ± 21% in men under 25 years to 25 ± 18% in the oldest group.[247] The only parameter to actually increase with age was sperm concentration, which was 62 ± 44 million/ml in men <25 and

95 ± 103 million/ml in men over 55. However, whether this effect was directly due to age per se is not clear given that the older subjects also had a smaller semen volume and a longer duration of abstinence, both of which tend to increase sperm concentration. In contrast to sperm concentration, total sperm counts decreased with age and were 24% lower in the oldest versus the youngest age group. These data suggest that changes in semen parameters are likely to contribute to the decline in fertility in men with aging, but given the relatively modest magnitude of these changes, other factors including a decline in sexual activity are likely to be as, if not more, important.

FSH and Sertoli Cell Regulation

Longitudinal studies of men show a rise in circulating FSH levels with aging.[14,16,248] Interestingly, although the major negative feedback regulator of FSH in men is inhibin B,[17] cross-sectional studies indicate that levels of this gonadal peptide are only modestly lower in older than younger men.[249,250] Nonetheless, inhibin B levels show a strong inverse correlation with FSH and a strong positive correlation with testicular volume in older men.[249,250] Rigorous histomorphometric analysis of the testes of men who have died suddenly have shown a decrease in the Sertoli cell population with aging but no age-related decline in the number of germ cells per Sertoli cell, implying that there is a significant relationship between sperm production rates and the number of Sertoli cells.[251] These data suggest that the moderate reduction in testicular size with aging reflects a decrease in Sertoli cell mass, with relative preservation of Sertoli cell function (as evidenced by serum inhibin B levels) due to the compensatory increase in FSH secretion. This relationship is somewhat analogous to the state of compensated hypogonadism described later where normal Leydig cell secretion of testosterone is maintained by a compensatory increase in LH secretion.

Steroidogenesis (Epidemiological Studies)

Reproductive aging in men is characterized by a gradual and variable decline in serum testosterone levels from the fourth decade onwards that is quite distinct from the complete cessation of gonadal function that occurs at menopause. In the last 15 years or so, the publication of several large longitudinal cohort studies of American,[14–16] Australian,[252] and European men[248] has helped to provide some clarity in this controversial area, showing a reduction in total testosterone levels of 1–2%/year from the fourth decade onwards. In contrast, levels of the major binding protein for testosterone, sex hormone binding globulin (SHBG) increase by ~1.3–2.5%/year with age[14–16,252] such that the decrease in free testosterone levels is greater than that of total testosterone and is typically in the range of 2–3%/year. One cross-sectional study of men over the age of 70 showed that total testosterone levels remained stable with age, while free levels showed a significant decline.[253] The clinical significance of the disproportionately greater decrease in free testosterone levels with aging has not been established. The traditional teaching has been that it is only the free and bioavailable testosterone fractions that are biologically active. However, the demonstration that megalin, a receptor in reproductive tissues, can promote the cellular uptake of testosterone bound to SHBG through a process of endocytosis has thrown this free hormone hypothesis into question.[254] While the debate continues as to whether testosterone bound to SHBG is biologically active, Endocrine Society clinical practice guidelines recommend measurement of free or bioavailable testosterone in conditions associated with altered SHBG levels.[255]

Data on the impact of aging on the two major metabolites of testosterone (T), dihydrotestosterone (DHT) and estradiol, are more limited. The only large study to examine longitudinal changes in total DHT was the Massachusetts Male Aging Study (MMAS), which showed an increase of 3.5%/year.[16] However, in cross-sectional analyses, total DHT levels showed no age trend in either the MMAS cohort[16] or those of other studies.[256,257] The reason for the preservation of DHT levels in the face of decreasing levels of T, its major substrate, does not appear to be an increase in the activity of 5α-reductase as studies conducted in skin fibroblasts[258] and the prostate[259] show an actual decrease in 5α-reductase activity with age. Unlike testosterone, most studies show no change in estradiol levels with age,[248] although some show a decrease particularly in the levels of free estradiol.[260,261]

The clinical significance of the age-related change in testosterone is unclear for a number of reasons. First, the decline in testosterone is gradual, and a significant number of older men remain eugonadal. Second, even in the cohort of older men whose testosterone levels fall below the normal range for healthy young men, the threshold at which symptoms of androgen deficiency develop and adverse health outcomes ensue is uncertain and may differ for different target tissues.[262] Third, while epidemiologic studies show correlations between low testosterone levels and a variety of physical, cognitive, and metabolic endpoints, clinical trials of testosterone supplementation have yielded inconsistent results, and there is ongoing uncertainty concerning the long-term risk:benefit ratio.[263] While use of testosterone has increased dramatically in the last two decades in large part due to aggressive direct to consumer advertising, recent studies suggesting that testosterone therapy may increase the risk of cardiovascular events in men is likely to reverse this trend.[264–266]

In young healthy males, the reference range for testosterone is typically 300–1000 ng/dl depending on the assay methodology used. Estimates of the prevalence of

hypogonadism in aging men vary significantly depending on how the condition is defined. Using a purely biochemical definition based on plasma testosterone level alone without any reference to clinical symptoms, a prevalence of 20–50% has been reported depending on the age of the cohort and the predefined testosterone cutoff.[15,267] In contrast, the prevalence of hypogonadism when assessed by symptomatic androgen deficiency in combination with an arbitrary testosterone cutoff of <300 ng/dl is significantly lower at 5.6%.[267] When the diagnosis is further refined by systematically determining in a large population the testosterone threshold below which symptoms become increasingly prevalent, only 2.1% of middle-aged and elderly men meet criteria for hypogonadism, although this number increases with increasing age, obesity, and comorbid illness.[268] It is clear, therefore, that the potential exists to overdiagnose hypogonadism in older men and in some cases institute treatment that may be neither necessary nor beneficial. Thus, recent guidelines recommend that a diagnosis of hypogonadism only be made when consistent signs (e.g., gynecomastia, small testes, decreased body hair, reduced muscle mass, increased body fat) and symptoms (e.g., decreased libido, erectile dysfunction, decreased energy levels, low mood, poor concentration and memory) are present in association with unequivocally low testosterone levels.[255] Because of the considerable biologic variability in testosterone levels within subjects over time,[269] the diagnosis of age-related hypogonadism should never be based on a single blood sample as up to one-third of low levels are normal when repeated.

Patients with classical hypogonadism are traditionally classified as having either primary hypogonadism due to testicular failure or secondary hypogonadism due to hypothalamic-pituitary dysfunction.[255] However, the changes in the male HPG axis with aging appear more complex in that they involve multiple levels (Figure 37.8) and are significantly modified by other factors such as obesity and comorbidity. The situation is further complicated by the fact that a significant proportion of men with elevated gonadotropins continue to have normal testosterone levels.[270,271] Wu and colleagues recently proposed that this hormonal milieu could be viewed as a state of compensated hypogonadism, analogous to subclinical hypothyroidism, with the potential to progress to overt hypogonadism over time.[272] In an analysis of a large cross-sectional cohort of community-dwelling men (aged 40–79 years) in the European Male Aging Study (EMAS), they classified subjects into four categories of gonadal status using a testosterone threshold of 303 ng/dl and an LH of 9.4 U/l. The four categories were: eugonadal (T ≥ 303 ng/dl and LH ≤ 9.4 U/l); secondary hypogonadism (T < 303 ng/dl and LH ≤ 9.4 U/l); primary hypogonadism (T < 303 ng/dl and LH > 9.4 U/l); and compensated hypogonadism (T ≥ 303 ng/dl and LH ≥ 9.4 U/l). The majority of men were eugonadal (76.7%), with 11.8% having secondary hypogonadism, 2% primary hypogonadism, and 9.5% compensated hypogonadism. Despite the rise in LH levels that has been reported with aging,[14,16,248] most of the men with low testosterone levels had secondary hypogonadism for which obesity was the strongest predictor with age having no significant effect. Only a minority of men had primary hypogonadism despite the fact that aging is associated with significant changes in Leydig cell function as discussed later. Primary hypogonadism most likely represents the purest form of late onset hypogonadism in that it has the strongest relationship

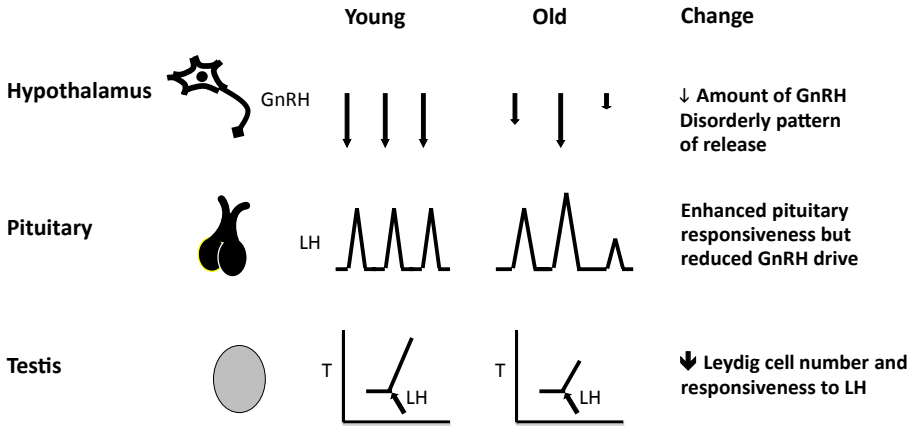

FIGURE 37.8 Changes in the hypothalamic-pituitary-gonadal axis with aging in the male. The arrows indicate that the amount of gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus decreases with age as evidenced by frequent sampling studies showing lower luteinizing hormone (LH) pulse amplitude, where LH is used as a surrogate marker of GnRH,[276] a more disorderly pattern of release,[277] and greater LH suppression after administration of a submaximal GnRH antagonist dose.[278] In contrast, there is evidence of heightened pituitary responsiveness to GnRH Kaufman.[279–281] Functional studies demonstrate an age-related reduction in the amount of testosterone secreted per LH bolus,[282,283] which correlates with the histological demonstration of a reduction in Leydig cell number with aging.[284]

with age with the prevalence increasing from 0.1% in the 40–49 age group to 5.4% in those aged 70–79 years. Compensated hypogonadism is also strongly associated with age reaching a prevalence of 21% in men over 70 years. Given the wide reference range for testosterone, Wu et al. hypothesized that these men may have experienced a decrease in testosterone secretion within the normal range and that the high LH levels reflect a corresponding readjustment of the HPG feedback that partially restored the decline in Leydig cell testosterone secretion that occurred with aging. An alternative possibility is that although the testis is less responsive to LH stimulation, testosterone secretion in these men is unchanged because of an elevation in LH secretion due to a concomitant change in the set point of negative feedback at the hypothalamic-pituitary level.[273] However, as discussed, later studies on the impact of aging on testosterone-mediated negative feedback have not consistently shown this.[274,275]

While aging per se causes a modest decline in testosterone levels, data from several studies highlight that age-related increases in obesity and chronic illness as well as smoking and marital status have a significant impact on circulating testosterone levels.[16,270,285,286] In fact, the MMAS showed that obesity had a greater impact on testosterone levels than age alone and that a 4–5 kg/m^2 increase in body mass index (BMI) was associated with declines in total serum testosterone comparable to that seen with approximately 10 years of aging.[285] These data imply that the apparent age-related decline in testosterone may not be an inevitable consequence of the aging process but rather reflect health and lifestyle factors and thus be potentially preventable and/or reversible. This hypothesis was recently confirmed in a 4-year follow-up study of the EMAS cohort, which, similar to the longitudinal studies, showed that weight loss is associated with a significant rise in testosterone levels, while weight gain is associated with a corresponding decrease in testosterone levels.[287]

Aging in men is associated not only with a reduction in absolute levels of testosterone but also a blunting of the characteristic diurnal rhythm in the secretion of this steroid in young, healthy males.[288] The physiological significance of the diurnal rhythm in testosterone secretion is unknown.

Mechanism of Testosterone Decline with Age

Hypothalamus and Pituitary

As previously discussed, direct measurement of GnRH is not possible in the human due to its confinement to the hypophysial-portal circulation. However, frequent blood sampling studies performed in older men show lower LH pulse amplitude[276] and a more disorderly and chaotic pattern of LH release,[277] providing indirect evidence for a decrease in GnRH drive with aging (Figure 37.8). Others have employed a more direct approach to assessing GnRH secretion by measuring the degree of inhibition of endogenously secreted LH in response to increasing doses of a potent and selective GnRH antagonist in a cohort of men whose ages span the third to the eighth decades.[278] Increasing age was found to significantly potentiate the suppressive efficacy of any given antagonist dose on endogenously driven LH release consistent with a reduction in GnRH secretion in older men.

An alternative explanation for the reduction in LH pulse amplitude that is apparent in older men is that aging is associated with impaired pituitary responsiveness to GnRH. However, available evidence suggests that gonadotroph function does not change with aging in men. In fact, studies have shown that the LH response to GnRH is actually increased in older men (Figure 37.8), whether administered as a single bolus across a wide dose range[279,280] or as repetitive pulses through a portable infusion pump for 14 days.[281] Heightened gonadotroph responsiveness to small amounts of GnRH could potentially be due to upregulation of GnRH receptors, a hypothesis that cannot be directly addressed in the human.

Studies by Veldhuis and colleagues provide evidence for a key role of the ambient sex steroid milieu in regulating pituitary responsiveness. By examining the LH response to GnRH in young and old men before and after ketoconazole-induced androgen depletion, they showed that a hypoandrogenemic milieu sensitized gonadotroph response to GnRH stimulation disproportionately in the older men.[280] Thus, it appears that gonadotroph responses reflect important interactions between GnRH dose, testosterone concentrations, and age.

Human studies on the impact of aging on testosterone-mediated negative feedback have given quite conflicting results. Depending on the experimental paradigm employed, aging has been variably reported to either increase[274,275] or decrease[289,290] the negative feedback action of testosterone on GnRH and LH. Recent autopsy studies of hypothalamic samples from young and old men show morphological evidence of an age-related enhancement of kisspeptin signaling in the infundibular nucleus,[291] also known as the arcuate nucleus. While it has not been proven, it seems plausible that the enhanced kisspeptin signaling in older men is a consequence of a decline in the sensitivity of the *KISS1* neurons to androgen negative feedback.

Testis

Mechanistic studies of testicular size and steroidogenic function corroborate the age-related decline in testosterone demonstrated in epidemiologic studies. Studies using ultrasound have shown that the testicular

volume of men aged greater than 75 years is approximately 30% smaller than that of men aged 18–40 years (20.6 versus 29.7 ml).[249] Similarly, histological examination of the testes in autopsy specimens from men who died following trauma or myocardial infarction indicate that Leydig cell number is approximately 44% lower in men aged 50–76 years than those aged 20–48 years.[284] As histological examination showed no evidence that men with reduced numbers of Leydig cells had an increased number of other interstitial cells, this reduction in Leydig cell number with age appears to be due to cell degeneration and dissolution as opposed to dedifferentiation to other interstitial cell types.[292]

While Sertoli cell loss is a critical factor in determining age-related changes in sperm production, the reduction in Leydig cells only becomes a rate-limiting step when the complement of cells falls to fewer than 10% of that seen in young, healthy men.[293] In practice, such a profound reduction in testosterone is rarely encountered due to aging alone and typically requires a "second hit" either to the testis or to the hypothalamic-pituitary unit.

Functional studies have confirmed that the secretory capacity of the testis in response to both physiologic and pharmacologic stimuli is significantly lower in older than in younger men. Initial studies showed an age-related decline in the ability of the Leydig cells to secrete testosterone in response to stimulation with human chorionic gonadotropin (hCG).[294,295] However, the experimental design employed in these earlier studies has a number of limitations in that baseline LH concentrations were not matched, tending to be higher in the older than the younger men; the doses of hCG used were large and provided a pharmacologic stimulus compared to an endogenously secreted LH pulse; and, in addition, hCG has the potential to cause Leydig cell desensitization.[296] To overcome these methodological issues, Veldhuis and colleagues developed an LH clamp paradigm to determine the physiologic basis for the reduction in testosterone secretion with aging. They first used a GnRH antagonist to abolish endogenous LH secretion so that baseline LH levels were matched, and then stimulated Leydig cells with fixed pulses of recombinant human LH.[282,283] This more physiologic experimental paradigm showed that while basal testosterone secretion is unchanged across a span of five decades, there is an age-related decline in the amount of testosterone released per LH bolus.[282,283]

Reproductive Aging in Animals

Spermatogenesis

Histological studies of the testis in the Brown Norway rat have shown a reduction in Sertoli cell number and function, a decrease in seminiferous tubule volume and sperm content, and accelerated germ cell apoptosis with aging.[297] Aging has also been associated with a marked increase in the number of spermatozoa with abnormal flagellar midpieces as well as a reduction in the percentage of motile spermatozoa.[298] In addition, analysis of the ejaculate of old Wistar rats has shown a decrease not only in sperm count with age but also the percentage of spermatozoa developing progressive motility with the latter showing a substantial reduction at 24 months of age.[299] In contrast, available, albeit limited, data suggest that there is no difference in fertility between rats aged 2 months and rats aged about 17 months,[300] although advancing paternal age was associated with an increase in preimplantation loss.[301] Thus, male rats and men appear similar in the way that sperm quality is reduced with increasing age, but the consequences for fertility are relatively slight.

Steroidogenesis

Analysis of testosterone in fecal samples from wild baboons confirms an age-related decline analogous to the human,[302,303] but this effect may be confounded by variability in non-age-related factors such as predation and malnutrition. Studies performed in young and old rhesus monkeys show an enhancement of the diurnal rhythm in testosterone with age,[304] in contrast to the blunting of this rhythm that has been demonstrated in the human.[288] Although no significant age-related change in mean testosterone levels over 24 h was observed, a significant age-related difference in testosterone levels was evident between 1200 and 1600 h, with the older monkeys having much lower testosterone levels during the daytime.

Aging Effects on the HPG Axis

Hypothalamus and Pituitary

The impact of aging on the HPG axis in the rodent depends on the strain studied. As mentioned earlier, the Brown Norway rat has advantages over other strains in having unusual longevity without succumbing to obesity and cancer and is thus a useful model for studying the effects of aging without the confounder of illness. In vivo studies in the Brown Norway rat show a reduction in LH pulse amplitude similar to that seen in the human, while in vitro experiments demonstrate reduced basal and stimulated GnRH release by hypothalamic tissue.[297] While the number of GnRH neurons does not appear to decrease with aging, a reduction in the number of synaptic inputs to GnRH neurons has been observed,[305] as well as a reduction in GnRH mRNA and peptide.[297] These data therefore support a hypothalamic component to male reproductive aging in the rat similar to that seen in the human.

Testis

Rodent studies also confirm the age-related defect in Leydig cell function that has been observed in the human. Decreased sensitivity to individual LH pulses could

reflect either a reduction in LH receptor number or activation or a defect in post-receptor signaling. Studies in the aged male Brown Norway rat demonstrate decreases in testicular LH-driven cyclic AMP (cAMP) accumulation and in enzymes such as steroidogenic acute regulatory protein, which regulate the rate-limiting steps of steroidogenesis.[306,307] Neither the administration of exogenous LH to aging rats in vivo[308] nor the incubation of aged Leydig cells with LH in vitro was capable of reversing these defects,[309] indicating that deficits in LH secretion are unlikely to underlie the age-related decline in Leydig cell steroidogenesis. In contrast, culturing aged Leydig cells with a membrane-permeable cAMP agonist that bypasses the LH receptor-adenylyl cyclase cascade was capable of restoring testosterone production to levels comparable to those of young cells.[307] These experiments therefore provide evidence that inefficient LH signal transduction, leading to reduced cAMP production, is responsible for the reduced steroidogenesis that characterizes aged Leydig cells. It is possible that similar inefficiencies in LH signaling may underlie the states of primary and compensated hypogonadism seen in aging men.[270]

Free radical damage has been proposed as a potential mechanism for the reduction in cAMP generation in aged rat Leydig cells. This theory is supported by the demonstration of an age-related increase in the formation of reactive oxygen species by mitochondria[310] as well as an impairment of antioxidant defenses in aging Leydig cells.[311]

CONCLUSION

There are many questions that remain unanswered. The capacity to reproduce varies from the time of puberty to the end of life, with substantial sex differences in the timing of reproductive transitions, especially aging. In fact, why females undergo senescence so much earlier than males, especially in humans, is of great interest to biologists, sociologists, anthropologists, and others. The "grandmother hypothesis" has been proposed to explain the potential evolutionary and adaptive value for a woman to outlive her follicular complement. This concept, while controversial, suggests that a postmenopausal woman contributes to raising kin (e.g., grandchildren, nieces/nephews, etc.) and can share her long life of experiences with her social group.[312,313] The fact that men can reproduce to a much older age is consistent with natural selective pressures for the sex with the lesser investment in pregnancy, lactation, and (sometimes) parenting.

In women, ovarian aging per se is central to reproductive senescence. Studies to date suggest that both genetic and environmental factors play a role, alone or in combination, but such studies are in their infancy. Future research that seeks to understand the genetic and environmental factors that prolong the health of oocytes and their supporting structures or hasten their demise will be required to extend reproductive capacity to meet the social needs of women in industrialized countries. There is now evidence that neuroendocrine aging occurs in women as well as animals. It will be important to determine the degree to which modification of these changes can extend reproductive life. Markers of ovarian aging have been identified in infertility populations, but further studies will be required to determine if these markers can predict menopause in the general population. Finally, further studies on the effects of the loss of reproductive hormones on nonreproductive systems including the brain (cognition, sleep, vasomotor symptoms), metabolism, bone, and cardiovascular disease will be critical to healthy aging in women as will be development of individualized approaches to treatment that optimize risk and benefit based on genetic and environmental information.

Basic research on reproductive aging is hampered by limitations in the availability of aging animals; the high cost of maintaining and/or purchasing such animals; and morbidity and mortality associated with aging. Thus, alternative models have been developed such as ovariectomy or orchidectomy to model loss of gonadal hormones. While these experimental models are extremely important, they are typically conducted in young animals. Because young and aged animals (and humans) differ in both hormone-dependent and -independent manners, there is a great need to conduct experiments in age-appropriate models. Another experimental limitation in females is that few species experience menstrual cycles or undergo a true menopause, and those that do typically experience menopause relatively later in life than in humans. For example, in the rhesus macaque it has been estimated that females undergo menopause at the equivalent age of a 65- to 90-year-old woman,[209] compared to the typical age of 45–55 years in women. Thus, new models of menopause are needed that better approximate the hypothalamic, pituitary, and ovarian changes in women.

Aging has more modest effects on the HPG axis of men than women. The most significant changes occur peripherally at the level of the testis, with steroidogenesis being impacted much more than spermatogenesis. Further studies are needed to dissect the impact of age per se versus age-related illness on the HPG axis, as well as to determine the contribution of changes in semen parameters to the modest decline in fertility seen in older men. Further studies will be required to determine whether optimal aging in men is associated with age-related norms or is better achieved with replacement to levels in young men.

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CHAPTER

38

Embryo Implantation

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INTRODUCTION

An essential task of procreation is to diversify the genetic repertoire and select advantageous traits to pass on to offspring. Procreation in prokaryotes and some eukaryotes is more efficient than the sexual procreation exhibited by higher eukaryotes, especially in placental mammals. Fostering an offspring within the womb is a demanding task, requiring multiple regulatory safeguards at critical steps. Thus, reproduction in viviparous mammals has adapted a more complex and highly regulated system. The embarkment of a new life in eutherians first depends on the union between a sperm and an egg (ovum) resulting in successful fertilization; failure to achieve this union leads to their demise. A one-cell fertilized egg, hence termed "embryo", undergoes several mitotic cell divisions, ultimately forming a differentiated tissue called the blastocyst with two distinct cell populations: a pluripotent inner cell mass (ICM) and an outer layer of extraembryonic trophectoderm.[1] A reciprocal interaction between the blastocyst and the maternal uterus initiates the process of implantation, a process through which the vascular system of the embryo is subsequently brought into communication with the maternal circulation, leading to the establishment of a functional placenta and guiding the appropriate progression of pregnancy. Maternal resources filtered across the placenta with selective barrier properties nourish and protect the conceptus. Historically, the process of implantation was often referred to as nidation, the term originating from the word "nidus", meaning a nest or a breeding place, since the blastocyst implants in a specialized chamber (crypt) at the antimesometrial pole (Figure 38.1(A)).[2]

In a historical context, Corner in 1947 wrote that "…the uterine chamber is actually a less favorable place for early embryos than say, the anterior chamber of the eye, except when the hormones of the ovary act upon it and change it to a place of superior efficiency for its new functions."[3] Subsequently, it was realized that the establishment of pregnancy results from the culmination of an intimate relationship between the developing embryo and the differentiating uterus. However, although conceptually accepted, the nature of such two-way interaction between the blastocyst and the uterus is still a challenging question. Embryo–uterine interaction leading to implantation is only initiated when embryonic development is synchronized with the preparation of the uterus to the receptive state. Specifically, the embryo must develop to the blastocyst stage and gain implantation competency, and the uterus through steroid hormone–dependent changes must attain the receptive stage before successful implantation can occur.

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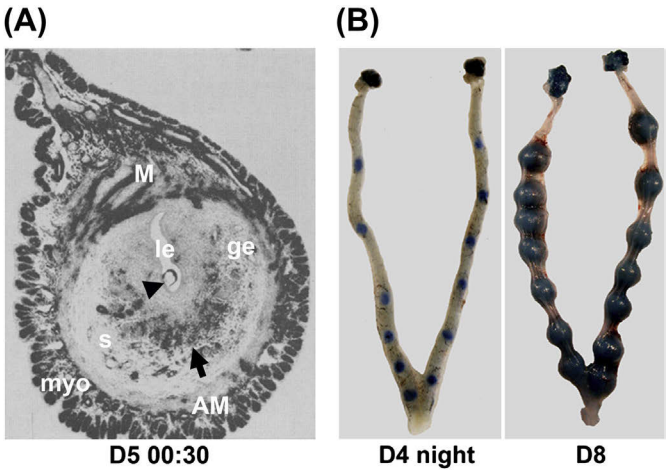

FIGURE 38.1 Increased endometrial vascular permeability upon attachment and implantation in the mouse. This figure is reproduced in color in the color plate section. (A) Transverse section of uterus on day 5 (D5) early morning (00:30) of pregnancy showing implantation at the antimesometrial pole within a crypt (arrowhead). This section also shows weak alkaline phosphatase activity in the uterine stroma (black, arrow). Note the edema in the outer areas of the mucosa, contrasting with the closely packed cells around the uterine lumen (presumptive primary decidual zone). Le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; M, mesometrial pole; AM, antimesometrial pole. *(Source: Reprinted with permission from Ref. 2.)* (B) Increased vascular permeability at the sites of blastocyst attachment with the uterine luminal epithelium was detected after an intravenous injection of a blue dye (Chicago Blue B6) solution in mice at midnight of day 4 (D4) and day 8 (D8) of pregnancy. On day 4, distinct blue bands (dark bands in this picture) indicate that the attachment process has been initiated.

The concept of uterine receptivity to implantation was first recognized by Alexander Psychoyos by utilizing embryo transfer experiments in pseudopregnant and delayed-implanting rodent models. Transferred blastocysts implanted only in uteri differentiated to a receptive stage.[4] Later, reciprocal embryo transfer experiments in delayed implantation mouse models, where blastocyst dormancy is maintained for many days by progesterone (P_4) treatment and reactivation is initialized with an injection of estrogen[a] (see the section Delayed Implantation (Mammalian Diapause)), found that blastocyst implantation competency is also a critical determinant for implantation in the receptive uterus.[5] A lack of synchrony between the embryonic development and the preparation of the uterus results in implantation failure.

PHYSIOLOGICAL AND MORPHOLOGICAL ASPECTS OF EMBRYO–UTERINE INTERACTIONS

Implantation is a prerequisite for subsequent development of the embryo. A significant number of pregnancy losses due to preimplantation embryonic death is common to many mammals and has been considered a selective process for the survival of superior embryos for implantation. However, dysregulated uterine events prior to, during, or after implantation are causes for poor pregnancy success rates in eutherians, either by immediate termination of pregnancy or by triggering adverse ripple effects that can be perpetuated through the remainder of pregnancy (see the section Adverse Ripple Effects Arising from Early Pregnancy Events). Therefore, a deeper understanding into preimplantation embryo development and implantation will help to identify aberrant mechanisms that can lead to unsuccessful or complicated pregnancy and provide strategies to improve infertility, prevent pregnancy complications, and/or develop novel contraceptive approaches to promote family planning. The achievement of uterine receptivity approaching attachment reaction is reflected in cellular and ultrastructural changes in the luminal epithelium, including gradual loss of cell polarity and the formation of microprotrusions from the apical surface called pinopodes.[6] The underlying mechanisms that coordinate blastocyst development to implantation competency with uterine receptivity are not yet fully understood.

Stages of Implantation

At the blastocyst stage, the trophectoderm of the developing embryo acquires competency to attach to the receptive uterine luminal epithelium that has been appropriately primed with the steroid hormones estrogen and P_4. If this condition of synchrony is met, an implantation-initiating adhesion cascade begins upon engagement of cell adhesion molecules at the luminal epithelial and trophectoderm surfaces (see the section Molecular Aspects of Embryo–Uterine Interactions). Many of these adhesion molecules transduce the signals necessary to sustain embryonic and maternal contributions to the formation of a placenta that supports fetal development through term.

Implantation begins when the blastocyst is placed in intimate physical and physiological contact with the uterine endometrium. Enders and Schlafke[7,8] classified the process of implantation into three stages: apposition,

[a] In this chapter, the term "estrogen" primarily includes estradiol-17β, estrone, and estriol, while estradiol-17β will specifically be abbreviated as E_2.

TABLE 38.1 Timing of Implantation in Various Species

Species	Day of Implantation	Ovarian Estrogen Requirement	Decidualization
Mouse	4.5[a]	Yes	Yes
Rat	5.5[a]	Yes	Yes
Hamster	3.5[a]	No	Yes
Guinea pig	5[a]	No	Yes
Rabbit	6.5[b]	No	Yes
Pig	13–14[b]	No	No
Cow	19–20[b]	No	No
Sheep	15–16[b]	No	No
Human	8[c]	?	Yes
Baboon	8[c]	?	Yes
Rhesus monkey	9[c]	?	Yes

[a] *Day 1, vaginal plug.*
[b] *Day 0, mating at estrus.*
[c] *Day 0, preovulatory estrogen or luteinizing hormone peak.*

adhesion, and penetration. Apposition is the stage when the trophectoderm cells become closely juxtaposed to the luminal epithelium. This is followed by the adhesion stage, when the association of the trophectoderm and the luminal epithelium is sufficiently intimate to resist dislocation of the blastocyst upon flushing the uterine lumen. The stage of penetration involves the invasion by the trophectoderm through the epithelium. Stromal cell transformation to terminally differentiated decidual cells (decidualization) is more extensive, and loss of the luminal epithelium is evident at this stage (see the section Molecular Aspects of Embryo–Uterine Interactions). These three stages of implantation are a continuous process over a period of time.

Apposition

In rodents, a generalized stromal edema occurs just prior to apposition. This event is thought to lead to uterine luminal closure and results in interdigitation of trophectoderm microvilli with those of luminal epithelia (apposition), followed by a closer contact between them (adhesion or attachment reaction). The luminal closure occurs throughout the entire uterus during pregnancy or pseudopregnancy, and thus this event does not necessitate the presence of blastocysts. Priming of the uterus with P_4 alone appears to be sufficient for this event to occur.

Attachment and Penetration

The attachment reaction is coincident with the localized increased stromal vascular permeability at the site of blastocyst contact as determined by intravenous injection of a macromolecular blue dye (uterine blue reaction) (Figure 38.1(B)).[4] The first sign of the attachment reaction in the process of implantation (day 1 = spermatozoa in the vaginal smear) occurs in the mouse and rat on the evenings of day 4 and 5, respectively, and day 6.5 in the rabbit.[4,9,10] In primates, the attachment reaction occurs approximately on day 8 in humans and baboons, day 9 in macaques, and day 11 in the marmoset.[11,12] In large domestic animals, the first signs of attachment occur on day 13 in pigs, day 20 in cows, day 16 in sheep, and day 19 in goats[13] (Table 38.1).

Although luminal closure and apposition occur in P_4-treated delayed-implanting mice, the attachment reaction fails under this condition. The superimposition of estrogen treatment is essential for this event. In mice, blastocysts are oriented with their ICMs directed toward the mesometrial pole, the site of entry of blood vessels into the uterus, while in humans the ICM is directed toward the antimesometrial pole. The mechanism by which the orientation of the blastocyst is achieved at the time of implantation remains elusive. There is evidence that in P_4-treated delayed-implantation mice, blastocysts are placed antimesometrially, and interdigitation (apposition) of luminal epithelial cell microvilli occurs with those of the abembryonic or lateral trophectoderm cells of the blastocyst with its ICM oriented toward the uterine lumen. This observation led to the suggestion that upon initiation of the attachment reaction and subsequent implantation process by estrogen, blastocysts retain the orientation they adopted during delay. During normal implantation in mice with the onset of luminal closure, blastocysts are placed at the antimesometrial side of the lumen along the uterine axis (Figure 38.1(A)). Shortly after the luminal closure, zona-encased blastocysts are located in implantation chambers with random orientation of their ICMs. However, with the beginning of the attachment reaction, blastocysts are correctly oriented with their ICMs directed at the mesometrial pole. This observation suggested that the trophectoderm of the entire blastocyst surface has the potential for attachment to the luminal epithelium, and that attachment occurs randomly immediately after the loss of zona pellucida. Evidence was presented to suggest that free movement of the ICM directs the correct orientation of the blastocyst. However, this issue still remains unsettled and warrants further investigation. All of these events from the luminal closure to the attachment reaction occur between about 86 and 92 h *post coitum* in mice.[14,15]

While implantation in mice occurs by displacing the luminal epithelium from the basal lamina to enhance trophoblast passage through the luminal epithelium into the stroma, humans exhibit an interstitial type of implantation in which blastocysts are embedded within

the subepithelial stroma. Ultrastructural studies show that implantation in humans is intrusive: trophoblast cells penetrate through the luminal epithelium and basal lamina into the stroma.[16] Although the significance of different implantation strategies adopted by different species is not clearly understood, one common feature is the increased endometrial vascular permeability at the site of blastocyst attachment in many animals studied. It is presumed that this increased permeability also occurs in the endometrial bed in human implantation, but this has not yet been clearly documented.

Penetration of blastocyst trophectoderm through the luminal epithelium and basal lamina into the stroma is required to provide nutrition to the developing embryo and establish a vascular relationship with the mother. This process varies considerably from species to species with respect to timing and cytological features.[16] In rodents, penetration of trophoblasts is usually followed by decidualization of endometrial stromal cells surrounding the implanting blastocyst, eventually embedding the embryo into the antimesometrial stromal bed.

Comparative Aspects of Implantation

On the basis of different types of blastocyst–uterine cell–cell interactions, Bonnet classified implantation into three categories: central, eccentric, and interstitial.[17] Central implantation occurs in mammals such as rabbits, ferrets, and some marsupials. In these species, blastocysts grow and extensively expand prior to implantation. In contrast, blastocysts of mice, rats, and hamsters are small and show modest expansion. In these species, an implantation chamber is formed by the invagination of the uterine epithelium, characteristic of eccentric implantation. In guinea pigs, chimpanzees, and humans, the implantation process is interstitial (i.e. blastocysts embed into the subepithelial stromal bed). Based on ultrastructural studies, Schlafke and Enders classified implantation into intrusive, displacement, and fusion types.[16] In intrusive types of implantation, such as in humans and guinea pigs, trophoblast cells penetrate through the luminal epithelium, reaching and extending through the basal lamina. The displacement type of implantation occurs in rodents, in which the luminal epithelium is freed of the underlying basal lamina, facilitating the spread of trophoblasts through the epithelium. The fusion type of implantation, in which trophoblast cells make connection with the luminal epithelium by forming symplasma, occurs in the rabbit. In many rodents, including mice and rats, implantation always occurs at the antimesometrial side of the uterus, whereas in some bats, implantation is mesometrial. The noninvasiveness type of implantation is observed in domestic animals such as pig, sheep, cow, and horse, and in wallaby.[18] Embryos in domestic animals maintain free-floating status longer than invasive conceptuses and become elongated up to 100 mm by day 12. This increase is due to the growth of the extraembryonic tissue, enabling an efficient avenue for receiving nutrition from uterine secretions until the attachment reaction occurs.

In sheep, the blastocyst enters the uterus on day 4 and hatches from the zona pellucida on day 9. After day 10, elongation of the blastocyst occurs and it develops first into a tubular and then to a filamentous form.[19] Starting on day 11, the spherical or slightly tubular blastocyst begins to elongate until it reaches a length of 25 cm or more by day 17 and resembles a long filament composed mainly of extraembryonic trophoblast.[20] Cellular and molecular mechanisms regulating blastocyst elongation are not well understood, but they may involve local production of prostaglandins (PGs) and growth factors. After day 14, the filamentous conceptus becomes immobilized in the uterine lumen. The first changes in the luminal epithelium occur on day 14 in both uterine horns.[21] In ruminants, distinct areas of projecting aglandular uterine mucosa called caruncles are involved in attachment reaction. The caruncles become edematous with a folded and depressed surface. Caruncular foldings are perhaps the first step in the formation of crypts that constitute the maternal side of the future placentomes, which receive hematotrophic nutrition for the conceptus.[22] Dome-like cytoplasmic protrusions also appear on the caruncular epithelial cells. On day 16, the trophectoderm begins to firmly adhere to the endometrial luminal epithelium. The interdigitation of the trophoblast and endometrial luminal epithelium is observed in both caruncular and intercaruncular areas of the endometrium. Trophectoderm adhesion to the epithelium progresses along the uterine horn, with completion around day 22.[23,24] Unlike sheep, attachment in horse and pig involves multiple sites covering most of the embryonic surface. In all of these ruminants, a notable decidualization is absent.

Hormonal Requirements for Implantation

In all eutherian mammals thus far studied, the uterus differentiates into a receptive state when the implantation-competent blastocyst engages in an effective two-way interaction to initiate attachment. This state of uterine receptivity lasts only for a limited time when the uterine milieu is conducive to support blastocyst growth, attachment, and subsequent events of implantation.[13,25–27] The master regulators that specify uterine receptivity are ovarian steroids: P_4, considered the "hormone of pregnancy", and estrogens.

These hormones are crucial for implantation in mice and rats, but ovarian estrogen is not essential for implantation in several species, including pigs, guinea pigs, rabbits, and hamsters; P_4 alone can support implantation.[25,28–32] Embryonic-derived estrogen is implicated in implantation in these species, and in fact, rabbit, pig, and horse blastocysts have the capacity to synthesize estrogens. The mouse embryo lacks aromatase, the enzyme for estrogen synthesis,[33] while rabbit blastocysts express the aromatase gene.[34] Whether preimplantation estrogen secretion by the ovary or embryo plays crucial roles in human implantation is not clearly understood.

The uterus is composed of heterogeneous cell types that respond uniquely to changing ovarian P_4 and estrogen levels. In mice and rats, the coordinated actions of P_4 and estrogen regulating uterine cell proliferation and/or differentiation in a spatiotemporal manner establish the window of uterine receptivity for implantation.[35] In adult uteri of these species, estrogen stimulates proliferation of epithelial cells, whereas this process in the stroma requires both P_4 and estrogen.[4,26,35] Similar steroid hormonal regulation of cell-specific proliferation and differentiation occurs during the peri-implantation period.[36] For example, on day 1 of pregnancy (vaginal plug) in mice, epithelial cells undergo proliferation due to preovulatory estrogen secretion. In contrast, rising P_4 levels arising from newly formed corpora lutea initiate stromal cell proliferation from day 3 onward, which is further stimulated by ovarian estrogen secretion on the morning of day 4. Coordinated effects of P_4 and estrogen then downregulate epithelial cell proliferation and initiate differentiation.[35] The preimplantation ovarian estrogen secretion is followed by localized increased endometrial capillary permeability at the site of the blastocyst upon attachment.[4,26,36] In pseudopregnant mice, the steroid hormonal milieu in the uterus is similar due to the presence of newly formed corpora lutea. Thus, the sensitivity of pseudopregnant uteri for implantation on days 1–4 is quite similar to that during normal pregnancy, and blastocyst transfer into a pseudopregnant uterine lumen during the receptive phase elicits normal implantation and decidualization.

In pigs, estrogen produced by the conceptus trophectoderm between days 10 and 15 of pregnancy acts on the endometrium and is considered essential for the establishment of pregnancy.[37] Estrogen seems to alter endometrial $PGF_{2\alpha}$ secretion in an endocrine manner (toward uterine vasculature) to an exocrine direction (toward the uterine lumen).[38] While $PGF_{2\alpha}$ from the uterus in the nonpregnant cycle is the principle signal for ovarian luteolysis, $PGF_{2\alpha}$ sequestered in the uterine lumen in the pregnant uterus is unavailable to exert luteolytic effects on corpora lutea. Additionally, increases in specific histotroph components occur in the lumen after the release of estrogens from the conceptus on day 11.[37,39] Placental estrogens also act on the epithelium in a paracrine manner and increase the expression of specific growth factors, which, in turn, act on the trophectoderm, stimulating cell proliferation and development.

The baboon has been studied as a nonhuman primate model to understand human implantation. Uterine receptivity and implantation in this species are classified into three phases. The first phase, between days 8 and 10 postovulation of the menstrual cycle, appears to be regulated by estrogen and P_4. The second phase is induced by blastocyst "signals" superimposed on the estrogen–P_4 primed receptive endometrium. This phase is associated with functional and morphological endometrial changes that are distinct from those observed between days 8 and 10 postovulation in a nonconceptual cycle. The third phase is initiated following attachment and implantation of the blastocyst. Evidence suggests that embryo-derived factors directly or indirectly influence endometrial receptivity and implantation in primates.[40,41]

In humans, the 28–30-day menstrual cycle begins with vaginal bleeding as a result of endometrial sloughing from P_4 withdrawal. A gradual rise in estrogen levels from maturing ovarian follicles then leads to extensive proliferation of the epithelium, stroma, and vascular endothelium, regenerating the endometrium and initiating the proliferative (follicular) phase. Numerous glands develop during this period, assuming tortuous morphology during the late proliferative phase. After ovulation, the luteal (secretory) phase commences with elevating P_4 levels from the newly formed corpus luteum. Glands become highly secretory with stromal cell differentiation ("predecidualization"). In the absence of a viable embryo, the corpus luteum undergoes luteolysis with a fall in P_4 levels, while an implanting blastocyst secretes chorionic gonadotropin, which maintains the corpus luteum to support pregnancy (see Chapters 23 and 28).

The impact of supraphysiological levels of estrogen on human endometrial receptivity, although controversial, has been investigated at the clinical level. In patients who respond excessively to gonadotropin stimulation (follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG)) for ovulation induction during in vitro fertilization (IVF) cycles, high serum estradiol levels (>3000 pg/ml) on the day of hCG administration are detrimental to uterine receptivity,[42] regardless of the number of oocytes retrieved or serum P_4 levels. In addition, an increase in serum estrogen levels during the preimplantation period has been observed in high-responder compared to normal-responder patients.[43]

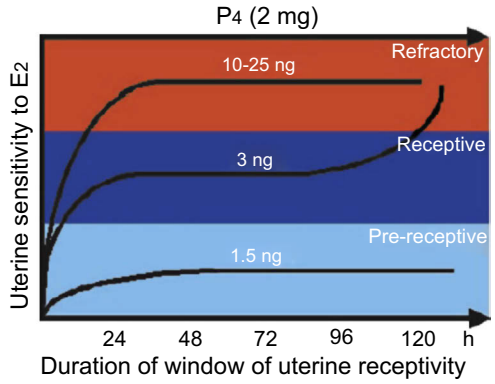

FIGURE 38.2 A scheme depicting modulation of the window of receptivity in the P4-primed uterus in response to changing estrogen levels in the mouse. This scheme shows that estrogen at a low threshold level extends the window of uterine receptivity for implantation, but higher levels rapidly close this window, transforming the uterus into a refractory state. *Source: Reprinted with permission from Ref. 5.*

Moreover, decreasing estrogen levels during the preimplantation period by a step-down FSH protocol increase implantation and pregnancy rates in high-responder patients.[44] There is also now evidence that E_2 at concentrations of $\geq 10^{-6}$ M in an in vitro culture induces a deleterious effect on embryonic adhesion to the substratum.[45] In mice, while lower estrogen doses maintain receptivity, higher doses transit the uterus to a nonreceptive phase (Figure 38.2).[5] Therefore, it may be possible to extend the window of implantation in humans by manipulating estrogen levels.[43,44]

Window of Implantation

Uterine sensitivity to implantation is classified as three different phases: prereceptive, receptive, and nonreceptive (refractory).[4,13] In mice, while the uterus is fully receptive on day 4 (the day of implantation), it is considered prereceptive on days 1–3 of pregnancy or pseudopregnancy, and by the afternoon of day 5, the uterus becomes refractory to blastocyst implantation. These phases have been defined by employing embryo transfer experiments in pseudopregnant rodents.[25]

Early work on uterine receptivity in mice demonstrated that the uterus in this species is only rendered receptive if exposed to estrogen 24–48 h after P_4 priming.[46] However, it is now known that the window of receptivity can be prolonged for an extended period by providing a low dose of estrogen in mice. Using an experimentally induced delayed implanting mouse model, a low dose of estrogen (3 ng/mouse) postpones uterine refractoriness to implantation for at least 4 days, while a higher dose of estrogen (10–25 ng/mouse) causes rapid closure of the receptive phase. Uterine nonreceptivity induced at high estrogen levels is accompanied by aberrant uterine expression of implantation-related genes.[5] This does not occur in normal implantation, presumably due to a small of amount of preimplantation estrogen secretion on day 4 of pregnancy. Using different genetic mouse models, it was shown that a short delay in the initial attachment reaction in mice during pregnancy creates adverse ripple effects resulting in developmental anomalies during the subsequent course of pregnancy.[47–51] These results suggest that careful regulation of estrogen levels is one of the important factors for improving implantation rates in IVF and embryo transfer programs.

Another critical factor determining the window of implantation is the blastocyst's state of activity (implantation competency). In the delayed implantation model in rodents, blastocysts undergo zona hatching, albeit at a slower pace, but they become dormant without initiating the attachment reaction even after P_4 priming of the uterus. However, a single injection of estrogen promptly initiates blastocyst activation and implantation in the P_4-primed uterus.

The process of implantation is considered a proinflammatory reaction, and increased vascular permeability at the site of blastocyst implantation is common to many species. Thus, it was suggested that histamine plays a role in implantation and decidualization.[52] Earlier observations suggested that uterine mast cells were the possible source of histamine, and its release from mast cells by estrogen was important for implantation.[53,54] This suggestion was based on the observations that local histamine application stimulates uterine hyperemic and edematous responses.[55] Furthermore, a histamine antagonist pyrathiazine or an inhibitor of histidine decarboxylase (HDC) was shown to interfere with implantation when instilled into the uterine lumens of rats and rabbits.[53,56]

Histamine works via at least four histamine receptor subtypes (H_1–H_4),[57–59] and blocking of both H_1 and H_2 receptors was shown to interfere with implantation in rats.[60] Subsequent studies also showed that histamine induces implantation in delayed-implanting rats when injected with a suboptimal dose of estrogen.[61] However, uterine mast cell numbers and histamine content are reduced both after estrogen treatment and during implantation,[52] and successful implantation and birth of live offspring in mast cell–deficient mice and other evidence have instead suggested that uterine mast cell histamine is not essential for implantation.[62,63] Thus, if histamine is involved in implantation, it should be provided either by major uterine cell types or by embryonic cells. While mouse blastocysts do not have the capacity for histamine synthesis,[64] HDC is expressed in uterine epithelial cells on day 4

of pregnancy in mice prior to implantation, but not in decidual cells.[65] In addition, H_1–H_3 receptor subtypes are not detectable in the uterus, but H_2 receptors are expressed in preimplantation mouse blastocysts. These observations as well as the inhibition of blastocyst zona-hatching and implantation by H_2 antagonists and an HDC inhibitor suggest that uterine histamine targets the blastocyst for implantation.[64] However, apparently normal implantation occurs in mice lacking HDC or H_2-type histamine receptor genes, suggesting the possible involvement of other vasoactive agents with overlapping functions or compensation by other histamine receptor subtypes upon deletion in this process.[58,66]

Although estrogen is essential for blastocyst activation and implantation in the P_4-primed mouse uterus, the mechanisms by which estrogen initiates these responses remain elusive. Evidence suggests that estrogen actions in uterine preparation and blastocyst activation for implantation are two distinct events. Indeed, embryo transfer experiments in delayed implantation recipient mice provide evidence that while E_2 initiates uterine events for implantation, catecholestrogens produced in the uterus from E_2 participate in the activation of dormant blastocysts toward implantation.[67] Blastocyst activation by catecholestrogens involves cyclooxygenase 2 (COX2)-derived PGs and cyclic adenosine monophosphate (cAMP).[67] These results provide evidence that both estradiol-17β and catecholestrogens are required for embryo–uterine interactions for successful implantation and that implantation occurs only when uterine receptivity coincides with the blastocyst's state of activity. Whether catecholestrogens have any role in implantation in other species is not known. Molecular pathways that are potentially involved in uterine receptivity and blastocyst activation are discussed in more detail in the see the section Molecular Aspects of Embryo–Uterine Interactions.

Endometrial Glands and Their Secretions

All mammalian uteri contain endometrial glands that synthesize, secrete, and transport a complex array of proteins and other factors termed histotroph.[22,68,69] The histotroph is a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and others. The idea that uterine secretions nourish the developing conceptus was discussed by Aristotle in the third century BC, and William Harvey in the seventeenth century. In 1882, Bonnet concluded that secretions of uterine glands are important for fetal well-being in ruminants.[70]

Evidence from primate and nonprimate species during the last century supports an unequivocal role for secretions of endometrial glands as primary regulators of the production of pregnancy recognition signals, implantation, placentation,[71–75] and conceptus survival and development. Studies on the uterine gland knockout (UGKO) ewe model, generated by continuous administration of a synthetic, nonmetabolizeable progestin in neonatal ewes from birth to postnatal day 56, reveal an essential role for endometrial glands and their secretions in normal estrous cycles and in peri-implantation conceptus survival and growth.[76–78] These ewes do not exhibit normal 17-day estrous cycles due to the inability of the uterus to produce sufficient luteolytic pulses of $PGF_{2\alpha}$. The lack of superficial or ductal glandular epithelium, coupled with an overall reduction in surface area of the luminal epithelium, reduces the numbers of oxytocin receptors that could respond to oxytocin, a luteolytic agent[77,79] (see Chapter 27).

Exogenous $PGF_{2\alpha}$ induces luteolysis in UGKO ewes, and they display normal estrus mating behavior.[76,78,80] However, adult UGKO ewes are unable to establish pregnancy, and the transfer of normal hatched blastocysts into the uteri of timed pregnant UGKO recipient ewes fails to rescue this defect.[76,77,81] Morphologically, normal blastocysts are present in uterine flushings of bred UGKO ewes on day 6 or 9 post mating, but not on day 14.[76] On day 14, uterine flushings of mated UGKO ewes contain either no concepti or severely growth-retarded tubular concepti.[81] Normally, the rapid elongation of the conceptus from a tubular to a filamentous form during the peri-implantation period in sheep (between days 11 and 13) is associated with the production of interferon tau (IFNτ, the type I IFN family member), the signal for maternal recognition of pregnancy that acts in a paracrine manner on the endometrial epithelia to inhibit the development of the luteolytic machinery.[79,82] Although the growth-retarded concepti in mated UGKO ewes produce little or no IFNτ, the endometrium of UGKO ewes nonetheless responds to intrauterine infusions of recombinant ovine IFNτ with increased expression of P_4-dependent IFNτ-stimulated genes, such as Wnt7a, which signals pregnancy recognition through endometrial factors.[79,83–85]

IFNτ regulates expression of a number of IFN-stimulated genes (ISGs) differentially in uterine epithelium and stroma, which are considered to play roles in endometrial differentiation and conceptus implantation in sheep.[86] The type I IFN receptor subunits, IFNAR1 and IFNAR2, are expressed in all major endometrial cell types, with the highest expression found in the luminal epithelium.[87] However, the majority of ISGs are induced in response to the conceptus or IFNτ only in the uterine stroma and deeper glandular epithelium, which is apparently due to the expression of IFN regulatory

factor 2 (IRF2), a potent repressor of gene transcription, in the superficial epithelium.[88–92] IFNτ acts in a paracrine fashion on uterine luminal and glandular epithelial cells to suppress transcription of ERα and oxytocin receptor genes,[93,94] thereby preventing development of the endometrial luteolytic mechanism. Therefore, the actions of IFNτ may not be completely limited to inhibition of luteolysis but may also be involved in inducing genes important for conceptus growth and uterine receptivity.

DELAYED IMPLANTATION (MAMMALIAN DIAPAUSE)

Delayed implantation (embryonic diapause) occurs when the embryo achieves a state of suspended animation with temporary arrest in blastocyst growth and metabolic activity within a synchronously quiescent uterus without implantation. Nearly 100 mammals in seven different orders have been shown to undergo delayed implantation.[95,96] In many species, the developmental arrest during diapause occurs at the blastocyst stage. Likewise, the endocrine landscape that physiologically governs embryonic diapause is represented by diverse strategies in different species.[97] Two functionally distinct categories of embryonic diapause are known.[96] Facultative diapause is best observed in rodents and marsupials (wallabies and kangaroos) characterized by developmental arrest induced by maternal conditions, including lactation, insufficient diet, shortage of drinking water, and other forms of stress.[96] It is believed to be a mechanism for synchronizing parturition with favorable environmental conditions for neonatal survival.[97] The second category is obligate diapause and can occur during every gestation period of a given species (Figure 38.3).

Obligate diapause is prevalent in mustelids, bears, seals, and some wallabies.[96] In most mammals displaying discontinuity of embryonic development, the progression to the blastocyst stage and postimplantation development follow a preordained, species-specific program. Species within the mustelid family display periods of embryonic diapause that are variable between

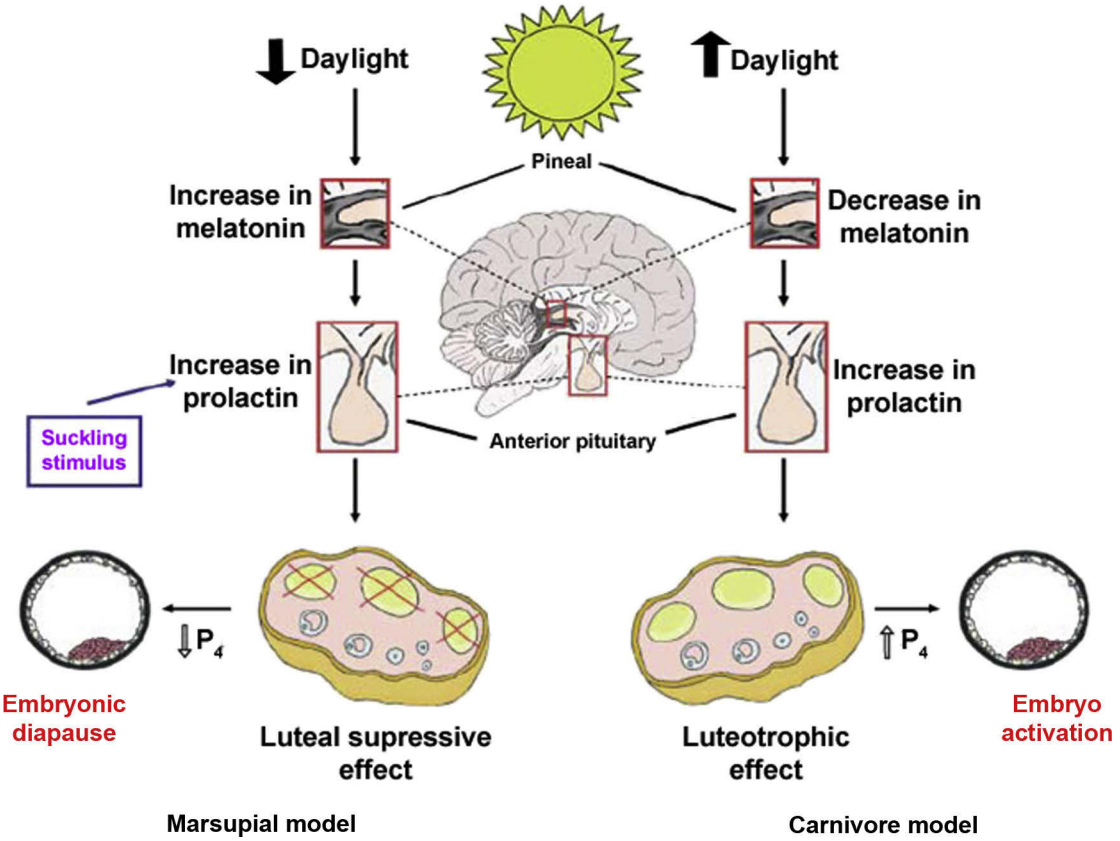

FIGURE 38.3 **Strategies for embryonic diapause for marsupials and carnivores.** In the marsupial model, which exhibits both obligate and facultative delay, suckling stimulus and increased melatonin secretion associated with nocturnal periods in excess of the summer solstice upregulate prolactin, which then inhibits luteal activation, thereby initiating and maintaining diapause. In the carnivore model, which exhibits obligate delay, the photoperiod associated with the vernal equinox decreases melatonin secretion, releasing prolactin from inhibition. Prolactin activates the corpus luteum (CL), provoking release of progesterone (P_4) and other factor(s) that terminate diapause. *Source: Reprinted with permission from Ref. 96.*

individuals but can be in excess of 350 days in the fisher (*Martes pennanti*), or be as brief as 3 weeks in the mink (*Mustela vison*).[96,98] Delayed implantation has been shown not to occur in certain species, including hamsters, rabbits, guinea pigs, or pigs. However, a recent interspecies embryo transfer study reports that embryos from nondiapausing sheep can undergo dormancy when transferred to a delayed implantation mouse uterus, and could subsequently be reactivated to produce normal offspring upon transfer back into the donor uterus.[99] This study concludes that embryos from a species deemed incapable of undergoing diapause are competent to enter embryonic diapause if the uterine environment favors this event, suggesting that all mammals inherently possess the capacity for embryonic diapause under the appropriate direction from maternal signals. Whether humans and their close primate relatives are capable of delay is under debate.[100]

Environmental, Neural, and Pituitary Regulation of Embryonic Diapause

Seasonal cues synchronize reproductive events in mammals, and it was recognized early on that photoperiod plays an important role in the termination of diapause and subsequent induction of implantation.[98] The lengthening of days prior to and after the vernal equinox influences the timing of implantation in numerous species, including the spotted skunk (*Spilogale putorius*)[101] and the mink.[96] Day length—or, more precisely, a regime of photoperiod in which minks are exposed to light during a critical period from 12 to 16 h after dawn—provides a facultative signal that induces implantation.[102] Melatonin produced by the pineal gland is considered the primary mediator of the photoperiodic regulation of diapause.[103] The essential factor regulated by melatonin proved to be prolactin, as implantation could be advanced by treatment with prolactin[104] or dopamine antagonists[105] (Figure 38.3), but further delayed by dopamine agonists.[104] Indeed, prolactin alone induces implantation in hypophysectomized mink,[106] and prolactin overcomes the effects of chronic melatonin treatment on the termination of diapause.[103] Prolactin executes its effects directly on the mink corpus luteum.[107] Although functional prolactin receptors are present in the mink uterus,[108] it is not known whether prolactin has a role at the level of the endometrium. Following ovulation, the ovarian follicle collapses and forms the corpus luteum.[109] The corpus luteum undergoes a remarkable structural reduction in size as diapause ensues, secreting low levels of P_4.[97,107] In contrast to the pattern of terminal differentiation that characterizes corpus luteum development in most species, the mink corpus luteum retains its mitotic potential during the period of diapause.[110] In response to the pituitary prolactin signaling that terminates diapause, the corpus luteum is rejuvenated, a process characterized by a several-fold increase in corpus luteum volume and in P_4 output.[107] As in other species, P_4 is essential for implantation and for the maintenance of gestation in mustelids. Nonetheless, numerous attempts to induce precocious implantation during diapause with P_4 or P_4 in combination with estrogens have been unsuccessful, suggesting that luteal factors other than P_4 are required for successful implantation in this species.[105] In the ferret (*Mustela putorius*), a mustelid species that does not undergo delay, a luteal protein factor has been shown to be necessary for implantation to occur.[111] Further studies have identified a secretory form of glucose-6-phosphoisomerase, also known as autocrine motility factor, as the luteal protein required for implantation in the ferret.[112] Its role in other species with embryonic diapause has not yet been confirmed.

Ovarian Regulation of Diapause in Rodents

In mice and rats, ovariectomy prior to the presumed "estrogen surge" on the morning of day 4 of pregnancy results in implantation delay, and a state of dormancy is initiated in blastocysts within the uterine lumen.[4,113] This condition is referred to as delayed implantation, and can be maintained for many days by continued treatment with P_4. A single injection of estrogen in the P_4-primed delayed implantation mouse rapidly initiates implantation and blastocyst activation.[4,26,114] The primary estrogen required for the activation of blastocysts for implantation is mediated by 4-hydroxyestradiol (4-OH-E_2), a cytochrome P450 (CYP) 1B1-generated metabolite of E_2 known as catecholestrogen.[67] Since CYP1B1 in the mouse uterus converts E_2 into 4-OH-E_2, E_2 injection is sufficient to initiate implantation in vivo. In contrast, dormant blastocysts cultured in vitro require 4-OH-E_2 to be activated for implantation. Blastocysts then can develop to term when sufficient amounts of P_4 and E_2 are provided.[67,115] Lactational delay is induced when mice become pregnant with postpartum ovulation. A delay of 1–7 days in implantation occurs in most lactating mice, and the length of delay is tightly associated with the number of sucking pups.[116] Sucking induces the secretion of prolactin through the alterations in hypothalamic function, which in turn suppresses ovarian estrogen secretion. Thus, an appropriate dose of estrogen resumes implantation in lactational delay.[117]

Activation of dormant blastocysts appears to involve an "early response" to catecholestrogens, since dormant blastocysts transferred into delayed implanting recipient uteri within 1 h of E_2 administration show implantation, while similar blastocysts transferred beyond this 1 h period fail to implant.[67] These results suggest that a rapid response to estrogen in the uterus occurs that is critical to implantation. In contrast,

dormant blastocysts cultured in the presence of a catecholestrogen, but not estradiol, gain implantation competency, and upon transfer implant in pseudopregnant recipients well beyond the 1 h "window" of estradiol treatment. Similar results were also obtained by culturing dormant blastocysts in the presence of PGE_2 or a permeable analog of cAMP, suggesting that this effect apparently involves the PG signaling pathway.[67] For example, co-incubation of dormant blastocysts with a selective COX2 inhibitor and 4-OH-E_2 efficiently blocks their activation and implantation upon transfer to suitable recipients. This effect of the COX2 inhibitor was partially reversed by addition of PGE_2 to the culture media. The results strongly suggest that the action of 4-OH-E_2 on dormant blastocysts is mediated via the PGs, leading to an increase in intracellular cAMP levels. However, it is still a mystery how catecholestrogens mediate activation of blastocysts. Although nuclear ERα is present in both the active and dormant blastocysts,[118] dormant blastocysts do not respond to estradiol and fail to attain implantation competency in vitro. In contrast, dormant blastocysts do respond to a catecholestrogen 4-OH-E_2 and become implantation-competent in vitro. The ER antagonist ICI-182,780 fails to reverse this response, suggesting that nuclear ER signaling is not critical to blastocyst activation.[67] These observations are surprising in the light of findings that ERα, ERβ, and estrogen-responsive finger protein mRNAs are expressed in preimplantation embryos.[119,120] Examining the direct roles of estrogens and/or P_4 in preimplantation embryo function and how steroid hormone signaling in the embryo and uterus is coordinated for implantation require further investigation.

In the ovariectomy model, dormant blastocysts survive as long as several weeks in utero, but the survival rate and their developmental competency are inversely correlated with the length of dormancy.[121,122] Since dormant blastocysts can survive in the delayed implanting uteri without exogenous P_4 in mice, P_4 does not seem to be an absolute requirement for the survival and retention of dormant blastocysts, but may be required for the longevity of blastocyst dormancy and to maintain the quiescent state of the uterus.[121,123]

The Embryo in Diapause

The experimentally delayed implantation model of rodents has widely been used to study molecular and cellular aspects of embryonic dormancy[124] (see the section Delayed Implantation (Mammalian Diapause)). During diapause, embryos appear to undergo a slow expansion in the absence of mitotic proliferation and are "metabolically dormant".[124,125] DNA synthesis and cell proliferation stop within 2–3 days of delayed implantation, but a low level of RNA synthesis is maintained. In this model, estrogen injection reactivates dormant blastocysts within 12 h and implantation resumes by 18 h. The reactivation of development comprises a rapid increase in size, followed by renewal of cell proliferation within 48–72 h. There is evidence[126] to indicate that fibroblast growth factor 4 (FGF4), produced by the ICM of the mink embryo, drives proliferation of the trophoblast cells prior to implantation.[127] In mice, terminal differentiation of the trophoblast is associated with endo-reduplication, where chromosomes replicate without proceeding through mitosis,[128,129] and there are some intriguing indications that such endocycles may also occur in mink trophoblast during its escape from diapause.[130] Another peculiarity of the carnivore blastocyst is that it remains encapsulated in the zona pellucida, which is breached at multiple sites by trophoblast outgrowths only at the time of implantation.[131] The progression of trophoblast into the endometrium does not differ greatly between species that exhibit diapause.[131,132]

A global gene expression analysis identified molecular pathways distinguishing blastocyst dormancy and activation in mice.[133] The major functional categories of altered genes include cell cycle, calcium signaling, adhesion molecules, and energy metabolic pathways. Thus, the two different physiological states of the blastocyst, dormancy and activation, are molecularly distinguishable in a global perspective. Heparin-binding epidermal growth factor (EGF)–like growth factor (HB-EGF) is an important mediator of embryo–uterine interactions during implantation.[134] Expression of this ligand is induced in activated blastocysts along with its potential receptors of the ErbB family,[133] supporting the importance of this signaling mediator in the initiation of implantation. EGF receptor (EGFR), COX2, and histamine type 2 receptor (H_2), the factors that are associated with blastocyst attachment reaction, are expressed in normal or activated blastocysts but are downregulated in dormant blastocysts (Figure 38.4).[27,64,123,135–137] In contrast, the G protein–coupled cannabinoid receptor CB1, which responds to endocannabinoids, is downregulated in activated blasto cysts, but remains upregulated in dormant blastocysts[138] (see the section Molecular Aspects of Embryo–Uterine Interactions). Collectively, these findings suggest that a complex molecular network regulates blastocyst dormancy and activation.

One of the intriguing features of dormant blastocysts is the activation of autophagy.[122] Autophagy, meaning self-eating, is a major cellular catabolic pathway by which macromolecules and organelles are recycled and used.[139] Dormant mouse blastocysts seem to utilize this pathway to prolong their survival in utero, and inhibition of autophagic pathway is associated with reduced blastocyst survival and compromised developmental competency in the ovariectomized model of delayed implantation in mice.[122]

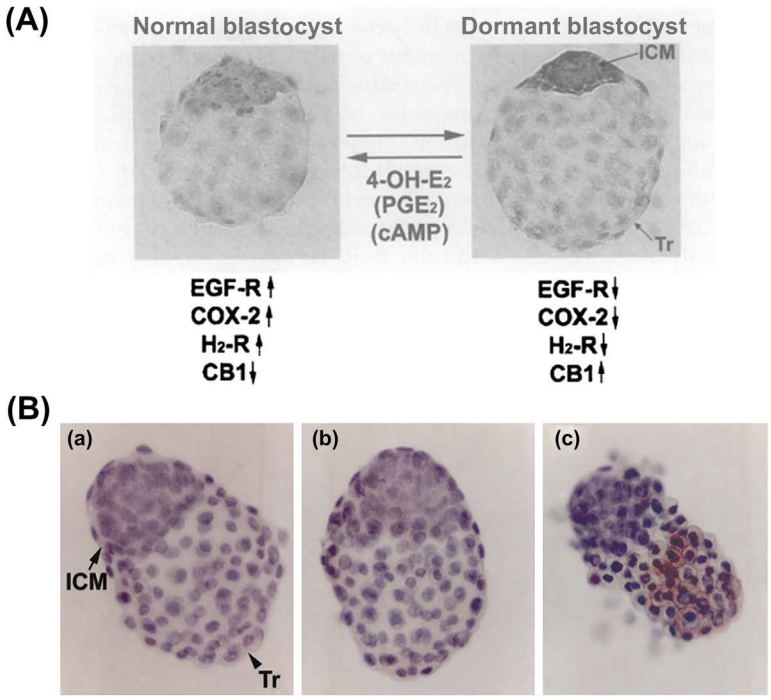

FIGURE 38.4 Molecular markers for blastocyst dormancy and activation. This figure is reproduced in color in the color plate section. (A) Although normal and dormant blastocysts apparently show morphological differences, several molecular markers are regulated reversibly by the blastocyst's state of activity. 4-OH-E_2, PGE_2, or cAMP can activate dormant blastocysts in vitro and either up- or downregulate specific marker molecules as indicated. Likewise, if normal blastocysts are induced to undergo dormancy by the use of delayed implantation in the uterus before the attachment reaction, expression of these markers is shifted in the reverse direction. EGFR, epidermal growth factor receptor; COX2, cyclooxygenase-2; H2R, histamine type 2 receptor; CB1, brain-type cannabinoid receptor. (B) Dormant blastocysts recovered from P_4-primed delayed-implanting mice on day 7 were cultured for 24 h in Whitten's medium, in the presence of (a) vehicle; (b) E_2; or (c) 4-OH-E_2. Blastocysts were processed for immunostaining and counterstained with hematoxylin. Red deposits indicate the sites of immunoreactive COX2. ICM, inner cell mass; Tr, trophectoderm. *Source: Reprinted with permission from Refs 67,137.*

Collectively, the delayed implantation models in mice and in other species could be exploited more extensively to better understand the molecular signaling that emanates from the uterus and influences blastocyst dormancy and vice versa.

Uterine Factors in Embryonic Diapause

As the host of the embryo during diapause and an ultimate place for implantation, the uterus plays a significant role in both the initiation and termination of discontinuous development in obligate delay. Indeed, embryos from nondiapausing sheep become dormant when placed in a mouse uterus under delay; upon transfer back into the donor ewe uterus, these embryos are reactivated and can produce normal offspring.[99] In addition, mink embryos in diapause co-cultured with uterine cell lines displayed the capacity to reinitiate embryonic development in vitro, providing further evidence that the uterus maintains diapause in this species.[140] The factors associated with inhibition of renewed development are not known. Histochemical evidence demonstrates that the transition of the mink uterus from delay to implantation is accompanied by increases in the quantity and distribution of glycosaminoglycans.[141] This concurs with observations of low levels of protein synthesis by the uterus during delay in the spotted skunk, followed by increased protein synthesis a few days prior to implantation.[142] Recent studies in mink have also identified a role for polyamine synthesis in embryonic diapause; polyamine deprivation in utero can induce the reversible arrest of embryonic cell proliferation.[143]

Studies of candidate gene expression during implantation in species with obligate delay have revealed patterns similar to those seen in the traditional rodent models. EGF and its receptor are present in the spotted skunk uterus during both the delay and postimplantation phases of gestation, and EGF activity is significantly elevated during the implantation process.[144] Leukemia inhibitory factor (LIF), a member of the interleukin 6 (IL6) family of cytokines essential for implantation in the mouse, is expressed in the uterine glands just prior to and after implantation in the mink.[145,146] LIF receptors were found in uterine glands and invading trophoblast during implantation in the spotted skunk.[147] COX2 (encoded by *Ptgs2*), another gene essential for mouse implantation, is not present in the uterus during diapause in the mink[148] or skunk uterus,[149] but is expressed in the trophoblast and endometrium of both species during implantation. Products of COX2 activity that subserve the implantation process are not well known, but prostaglandin E and D are implicated in the upregulation of transcription of the angiogenic factors associated with implantation.[96,150]

The *Msx* family of transcription factors is shown to play vital roles in fertility (see the section Molecular Aspects of Embryo–Uterine Interactions). Findings of persistent uterine *Msx1* during implantation delay and downregulation upon blastocyst activation, along with the findings that loss of uterine competency is correlated with missing *Msx1/Msx2* expression, also suggest a role of *Msx* genes in embryonic diapause.[151] Indeed, sustained uterine expression of *Msx* family members is

correlated with diapause in the mouse, American mink, and Australian tammar wallaby, with rapid downregulation with impending implantation.[152]

MOLECULAR ASPECTS OF EMBRYO–UTERINE INTERACTIONS

Although pregnancy events have always been a subject of curiosity, the field of reproductive physiology would not have progressed without the intellectual tenacity of pioneers in the field. Thoughtful and keen observations of early scientists greatly added to our understanding of reproductive biology. The current state of our knowledge of preimplantation and implantation physiology is the result of the accumulation of scientific observations gathered over many years.

Despite experimental success in initiating embryonic development outside the womb and in identifying numerous signaling molecules involved in the embryo–uterine dialog, there is still a significant knowledge gap in understanding the in vivo events of implantation. The successful implantation of an embryo is contingent upon cellular and molecular cross-talk between the uterus and the embryo. The coordination of the endocrine as well as cellular and molecular events via paracrine, autocrine, and/or juxtacrine factors acts in a dynamic manner to produce within the uterus a favorable environment, the receptive state, to support implantation.[153] The embryo also functions as an active unit with its own molecular program of cell growth and differentiation. Thus, deficiencies in uterine receptivity, embryo development, or the embryo–uterine dialog will compromise fertility.[153]

Implantation is a complex process involving spatiotemporally regulated endocrine, paracrine, autocrine, and juxtacrine modulators that span cell–cell and cell–matrix interactions.[153–155] However, the precise sequence and details of the molecular interactions involved have not yet been fully defined. Furthermore, the implantation process varies among species, thus precluding the formulation of a unified mechanism. In addition, ethical restrictions and experimental difficulties prevent direct analysis of embryo–uterine interactions during implantation in humans.

mRNAs encoding specific proteins are expressed in the uterus in a spatiotemporal manner and have helped to identify various genes with speculative roles in implantation.[153–155] Transgenic mouse models have furthered our mechanistic understanding regarding the roles of many genes in uterine receptivity and implantation, although the systemic deletion of many of these genes led to embryonic lethality, precluding the identification of their roles. The field was further advanced by the generation of conditionally deleted mouse models.[153] Despite this advancement, dynamic and overlapping expression patterns made it challenging to ascertain stage-specific roles. This section primarily focuses on the collective advances in the molecular basis of implantation in mice (Figure 38.5).[153]

Uterine Responsiveness to Hormone-Dependent Factors

Estrogen and P_4 effects in the uterus are primarily regulated by nuclear estrogen receptors (ERα and ERβ) and progesterone receptors (PRA and PRB). Differential uterine expression of ER and PR during the peri-implantation period in mice suggests that coordinated effects of estrogen and P_4 in uterine events for implantation are mediated via these nuclear receptors.[156] Furthermore, mouse models devoid of each receptor gene are informative as to how these receptors are involved in uterine biology.

ERα (encoded by the gene, *Esr1*) is the primary driver of estrogen action for uterine receptivity and implantation since *Esr1*$^{-/-}$ females are infertile.[157] However, further experiments using *Esr1*$^{-/-}$ mice showed that P_4 alone is sufficient to support decidualization (deciduoma) in response to artificial stimuli.[158,159] These results suggest that *Esr1*$^{-/-}$ mice have defective implantation, perhaps due to the failure of the attachment reaction but not due to the failure of decidualization events.[160] Microarray analysis of RNA from the *Esr1*$^{-/-}$ uterus also demonstrates a minimal response to estrogen,[161] suggesting that ERα is essential for proliferative and genomic responses of the uterus.

Female mice devoid of ERβ (*Esr2*) exhibit subfertility.[162,163] As in *Esr1*$^{-/-}$ or *Esr1*$^{-/-}$/*Esr2*$^{-/-}$ double-knockout mice, defects in the hypothalamic–pituitary–gonadal (ovarian) axis are apparent in *Esr2*$^{-/-}$ female mice.[163] The uterus of the *Esr2*$^{-/-}$ mouse retains full biological function, as demonstrated by uterine weight increase with E_2 administration.[162] Full genomic responsiveness to estrogen in these mice is also seen by microarray analysis.[161,162] Additionally, although *Esr2*$^{-/-}$ female mice exhibit subfertility, those that achieve pregnancy carry and deliver full-term pups, indicating that uterine function in terms of implantation, decidual response, and parturition is normal. These results point toward a minimal importance of ERβ in the adult uterus for estrogen response or pregnancy.

Mice deleted of progesterone receptor (*Pgr*$^{-/-}$) exhibit pleiotropic reproductive abnormalities, including impaired ovulation, uterine hyperplasia, and decidualization.[164] Selective ablation of the PRA isoform showed infertility with a milder phenotype, suggesting that PRA and PRB serve as functionally distinct mediators of P_4 action in vivo.[165] Experiments using both *Pgr*$^{-/-}$ and PRA$^{-/-}$ mice further reinforced the requirement of P_4 in decidualization.[164,165]

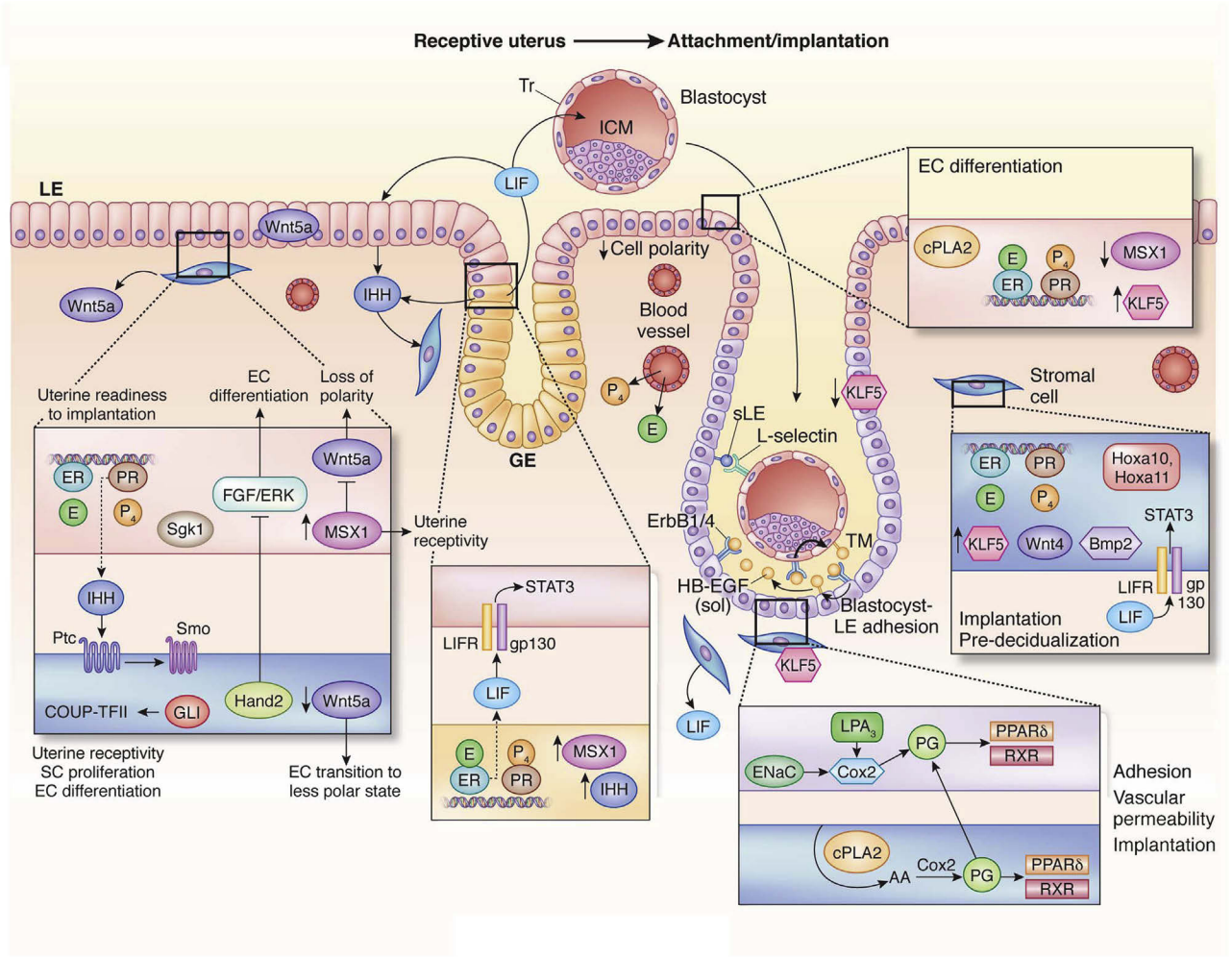

FIGURE 38.5 Signaling network for uterine receptivity and implantation. This figure is reproduced in color in the color plate section. This is a hybrid cartoon, based on mouse and human studies, portraying compartment- and cell type–specific expression of molecules and their potential functions necessary for uterine receptivity, implantation, and decidualization. Interplay of ovarian P_4- and/or E_2-dependent and P_4- and/or E_2-independent factors in the pregnant uterus in specific compartments contributes to the success of implantation in a juxtacrine–paracrine–autocrine manner. During attachment, interactions between the blastocyst and luminal epithelium (LE) involve ErbB1/4 and both transmembrane (TM) and soluble (Sol) forms of HB-EGF, as well as L-selectin ligands (sLE) expressed by the luminal epithelium to L-selectin receptors on the blastocyst. The other key signaling pathways for uterine receptivity and implantation are also shown. AA, arachidonic acid; BMP2, bone morphogenetic protein 2; cPLA2α, cytosolic phospholipase A2α; COUP-TFII, chicken ovalbumin upstream promoter transcription factor 2; Cox2, cyclooxygenase 2; E, estrogens; EC, epithelial cell (luminal and glandular epithelia); ENaC, epithelium sodium channel; ER, estrogen receptor; ErbB1/4; epidermal growth factor receptor 1/4; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; GE, glandular epithelium; gp130, glycoprotein 130; Hand2, heart- and neural crest derivatives–expressed protein 2; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Hoxa10/11, homeobox A10/11; ICM, inner cell mass; IHH, Indian hedgehog; KLF5, Kruppel-like factor 5; LIF, leukemia inhibitory factor; LIFR, LIF receptor; LPA3, lysophosphatidic acid receptor 3; MSX1, muscle segment homeobox 1; P_4, progesterone; PG, prostaglandin; PPAR-δ; peroxisome proliferator-activating receptor δ; PR, progesterone receptor; Ptc, Patched; RXR, retinoid X receptor; SC, stromal cell; SGK1, serum- and glucocorticoid-inducible kinase 1; Smo, Smoothened; STAT3, signal transducer and activator of transcription 3; Tr, trophectoderm; Wnt4/5a, Wingless-type MMTV integration site family members 4/5a. Compartment colors: blue, stroma; pink, luminal epithelium; orange, glandular epithelium; purple, epithelium at the attachment site. *Source: Reprinted with permission from Ref. 153.*

While both *Esr1*$^{-/-}$ and *Pgr*$^{-/-}$ mice show severe phenotypes of female reproductive failure, these mice have been used as model systems to study steroid hormonal regulation of several genes. Das and coworkers reported a non-ER-mediated estrogen signaling pathway that is resistant to an ER antagonist ICI-182,780 in *Esr1*$^{-/-}$ uteri.[160] Lactoferrin (LF) is a well-known estrogen-responsive gene in mice, and many natural or synthetic estrogens can induce this gene in wild-type uteri.[160] In contrast, while estradiol was not able to induce this gene in *Esr1*$^{-/-}$ uteri, a catecholestrogen 4-OH-E_2 was effective in this response. Since induction by 4-OH-E_2 was resistant to an ERα antagonist (ICI-182,780), ERα does not appear to mediate this effect. The possibility

that an alternative estrogen signaling pathway independent of nuclear ERs exists in the mouse uterus was further explored.[166] Using a differential display technique, it was found that several genes are upregulated or downregulated by estradiol and 4-OH-E_2 in both wild-type and *Esr1*$^{-/-}$ uteri independent of nuclear ERs. The upregulated genes were immunoglobulin heavy chain binding protein (Bip), calpactin I (CalP), calmodulin (CalM), and Sik-similar protein (Sik-SP), while the gene that encodes secreted frizzled related protein2 (SFRP2) was readily downregulated. An ER antagonist failed to neutralize these responses in both wild-type and ERα$^{-/-}$ mice, suggesting that these are ER-independent early responses. This provides compelling evidence that an alternative estrogen-signaling pathway is operative in the uterus. However, it is still unclear whether early estrogenic responses are mediated by a putative cell surface estrogen receptor[167] or by other nuclear receptors, such as ERγ or estrogen receptor–related receptors.[168,169] Alternatively, this could be explained by estrogen signaling via a membrane receptor of the G protein–coupled receptor family. Although several putative membrane progesterone receptors have been cloned,[170,171] whether similar types of membrane estrogen receptors are present in the uterus is not presently known.

ERα and PRA are expressed in all major uterine tissue compartments, and thus their compartment-specific contributions in uterine receptivity have been difficult to elucidate. Mice with epithelial cell-specific deletion of *Esr1* still show epithelial cell proliferation after estrogen exposure.[172] However, PR distribution between uterine compartments remained unchanged, suggesting a role for stromal ERα. Nonetheless, *Esr1*$^{-/-}$ females showed implantation failure with dysregulated expression of other estrogen-responsive genes, perhaps due to uterine failure to achieve receptivity. On the other hand, P_4 failed to attenuate estrogen-induced cell proliferation in females deleted of epithelial *Pgr*, suggesting a direct role of P_4 in inhibiting epithelial estrogen action.[173] These females are infertile with reduced expression of Indian hedgehog (*Ihh*) and LIF, resulting in poor uterine receptivity. This study also showed direct binding of PR to the *Ihh* promoter in the epithelium. However, these results are not consistent with those from a recombination study showing epithelial *Ihh* expression regulated by stromal PR.[174] Cell-specific expression of estrogen- and P_4-responsive genes in the uteri of these compartment-specific deleted mice would be worthwhile. Nevertheless, the results show that a reciprocal dialog between the epithelium and stroma is necessary for appropriate uterine receptivity and implantation.

Estrogen and P_4 execute their uterine functions by inducing and refining multiple paracrine, juxtacrine, and autocrine factors in a spatiotemporal manner. LIF is a mediator of estrogen function during implantation. LIF is critical for uterine receptivity because its deletion leads to implantation failure in mice.[175,176] By binding to its receptor, LIFR, and partnering with co-receptor gp130, LIF activates downstream signaling through signal transducer and activator of transcription 3 (STAT3).[177] This signaling is necessary for implantation as uterine deletion of *gp130* (*IL6st*)[151] or *stat3* (Sun et al., unpublished data) causes implantation failure. *Lif* expression is higher in the human endometrium at the time of implantation, but remains at lower levels in infertile women.[2,178,179] Whether LIF is required for human uterine receptivity and implantation requires further investigation.

Global deletion of tumor suppressor protein p53 (*Trp53*$^{-/-}$) showed small litters with inferior implantation and reduced *Lif* expression on day 4.[180] However, *Trp53*$^{-/-}$ mice show a range of phenotypes including exencephaly, especially in females, and widespread cancer at early reproductive age.[181,182] Males have also been shown to have compromised spermatogenesis.[183] Therefore, these mice may not be ideal to study reproduction. In contrast, another study using mice with uterine deletion of *Trp53* (*p53*$^{d/d}$) showed normal implantation but developed spontaneous preterm birth[184] (see the section Adverse Ripple Effects Arising from Early Pregnancy Events). These two studies cannot be compared directly because of the different approaches utilized (systemic versus conditional deletion).[180,184]

Many P_4-responsive genes participate in peri-implantation events in the uterus. The P_4-inducible immunophilin co-chaperone FKBP52 is required for optimal PR activity. *Fkbp52*$^{-/-}$ mice are infertile, with enhanced uterine estrogen-like signaling resulting from impaired P_4 responsiveness.[185,186] However, this P_4 resistance could be overcome with successful implantation and pregnancy by delivering excess P_4, depending on the genetic background of mice.[187] It was later shown that *Fkbp52*$^{-/-}$ mice are more vulnerable to oxidative stress due to diminished expression of a unique antioxidant *Peroxiredoxin 6* (*Prdx6*).[188] When exposed to oxidative stress such as paraquat, *Fkbp52*$^{-/-}$ mice showed implantation failure even with P_4 supplementation. This failure was reversed by supplementation with antioxidants. These data suggest that FKBP52 has more than one role in uterine biology. FKBP52 also plays a role in decidualization and endometriosis in mice and humans.[189,190] Steroid receptor co-activator 2 (SRC2, also known as *Ncoa2*) is also recruited by PR for its function.[191] Uterine deletion of *Ncoa2* led to pregnancy failure during the peri-implantation period due to defective progesterone action.[192] Since SRC2 is present in the human endometrium, it may play a role in optimizing P_4 function in human reproduction.[193]

P_4 signaling induces *Ihh* in the uterus, the deletion of which leads to implantation failure due to defective uterine receptivity.[194,195] *Ihh* is expressed in the epithelium

and interacts with its receptors patched and smoothened in the stroma, mediating stromal cell proliferation. These results implicate that Ihh executes epithelial–stromal interaction in a paracrine manner for uterine receptivity and implantation. In the human endometrium, expression of *Ihh* and its receptors is upregulated by progestins.[196] A proposed downstream target of Ihh is chicken ovalbumin upstream promoter–transcription factor (COUP-TFII, also known as *Nr2f2*) and is expressed in the subepithelial stroma.[197] Conditional deletion of *Nr2f2* in PR-expressing tissues leads to infertility due to implantation failure with excessive estrogenic signaling in the epithelium, suggesting that COUP-TFII participates in balancing ER versus PR activities. Paradoxically, stromal and myometrial deletion of *Nr2f2* deletion showed normal implantation with defective placentation. Incomplete uterine *Nr2f2* deletion is suggested as a cause for this phenotype discrepancy.[198]

Heart- and neural crest derivatives–expressed protein 2 (Hand2) is a P_4-induced stromal transcription factor that was shown to play a role in uterine receptivity and implantation in mice.[199] Mice missing uterine *Hand2* showed implantation failure with increased estrogenic activity and epithelial cell proliferation via FGF–ERK signaling. It was suggested that stromal Hand2 participates in uterine receptivity by altering epithelial differentiation. Whether the infertility phenotype in these mice could be reversed by excess P_4 administration or inhibitors of FGF-ERK signaling remains to be seen. A recent study also implicates Hand2 in decidualization.[200]

Fibroblast growth factor 7 (FGF7, also known as keratinocyte growth factor), is an established paracrine mediator of hormone-regulated epithelial growth and differentiation.[201] In all organs studied, FGF7 was uniquely expressed in cells of mesenchymal origin. Intriguingly, expression of FGF7 in the porcine uterus is exclusively restricted in the endometrial luminal epithelium and particularly abundant between days 12 and 15 of the estrous cycle and pregnancy.[202] Endometrial FGF7 mRNA levels are highest on day 12 in pregnant gilts and day 15 in cyclic gilts, and greater on day 12 of pregnancy than on day 12 of the estrous cycle. FGF7 protein was detected in the uterine flushings of both day 12 cyclic and pregnant gilts. The receptor for FGF7, known as FGF receptor 2 (FGFR2) or KGF receptor, is detected in both the endometrial epithelium and conceptus trophectoderm. Treatment of endometrial explants from day 9 cyclic gilts with estradiol increases FGF7 expression.[203] Furthermore, treatment of porcine trophectoderm cells with recombinant rat FGF7 increases their proliferation, phosphorylates fibroblast growth factor receptor 2 (FGFR2), activates the mitogen-activated protein kinase (MAPK or ERK1/2) cascade, and increases expression of urokinase-type plasminogen activator, a marker for trophectoderm cell differentiation.[203] Collectively, these results indicate that estrogen, the pregnancy recognition signal from the pig conceptus, increases uterine epithelial FGF7 expression and that FGF7, in turn, stimulates the proliferation and differentiation of the conceptus trophectoderm in the pig, which is the only species possessing a true epitheliochorial type of placentation except the camel.[202,203]

Uterine Responses to Factors Independent of Estrogen and P_4

Numerous genes crucial for uterine receptivity and implantation in mice are regulated by estrogen and P_4. However, there are transcription factors that are apparently not directly altered by these hormones but greatly influence uterine receptivity and implantation. *Msx1*, an ancient evolutionarily conserved homeobox transcription factor, plays a critical role in craniofacial development.[151] *Msx1* is also transiently expressed in the pregnant mouse uterine epithelium on the morning of day 4 of pregnancy, the day of uterine receptivity.[204] Expression then gradually disappears approaching the attachment reaction and remains undetected thereafter. These observations suggest that Msx1 is important for receptivity. Expression of *Msx2*, another member in the family, is very low to undetectable in the uterus during this time; however, *Msx2* is upregulated in *Msx1*-deficient uteri and exhibits a similar pattern to the expression of *Msx1* as observed in wild-type mice. This finding provides evidence for a compensatory role of *Msx2* in the absence of *Msx1*. Uterine deletion of *Msx* genes showed graded levels of infertility depending on single or double deletion.[151] Mice with uterine inactivation of *Msx1* ($Msx1^{d/d}$) produce small litter size or no litters, while mice with deletion of both *Msx1* and *Msx2* ($Msx1/Msx2^{d/d}$) show complete infertility due to failed or defective implantation with loss of uterine stromal *bone morphogenetic protein 2* (*Bmp2*) expression, and restricted *Ptgs2* expression in the luminal epithelium at the site of blastocyst apposition. Notably, mice with uterine inactivation of *Msx2* have normal fertility.[151] The implantation failure as noted in this study was later confirmed by another group.[205]

Msx1 expression is rapidly downregulated approaching implantation and thereafter, and it persists in delayed implantation uteri until implantation is induced by estrogen. Implantation largely fails in P_4-primed delayed implantation $Msx1^{d/d}$ uteri following estrogen treatment. These results suggest that *Msx* genes are critical for maintaining uterine readiness to implantation. *Lif* expression is downregulated in *Msx*-deleted uteri, but LIF supplementation cannot rescue implantation in deleted mice. The relationship between LIF and Msx remains to be explored.

Msx genes are likely to be involved in human implantation since they are downregulated in the endometrium

during the window of implantation similar to that which occurs in mice (reviewed in Refs 151,155). The luminal epithelium transits from a higher to lower polar state approaching blastocyst attachment.[206] This transition is absent in *Msx1/Msx2*$^{d/d}$ uteri at the anticipated time of implantation. Wnt5a, a noncanonical Wnt and a known mediator of cell polarity, is upregulated in the uterine epithelium and stroma in *Msx1*$^{d/d}$ and *Msx1/Msx2*$^{d/d}$ mice. In addition, the *Wnt5a* promoter shows Msx1 binding sites in a human uterine cell line. Further studies indicated that Wnt5a-β-catenin/E-cadherin signaling is a potential downstream target of Msx and participates in implantation by altering cell polarity with respect to adherens junctions.[151]

E-cadherin, a calcium-dependent adhesion molecule, participates in the formation of the epithelial adherens junctions in cooperation with α- and β-catenins.[207,208] E-cadherin is a critical factor for blastocyst formation, since its deletion leads to defective embryonic development resulting in failure to form the trophectoderm.[209,210] E-cadherin is implicated in uterine–embryo interactions because of its homotypic adhesive activity.[211] The components of the adherens junctional complex are expressed in the subepithelial stroma surrounding the implanting blastocysts, with apoptosis occurring in the luminal epithelium.[211,212] This junctional complex, in turn, forms a barrier surrounding the embryo, perhaps restricting passage of injurious stimuli from the maternal circulation. The results suggest that temporal and cell-specific expression of the adherence junction proteins in the uterus result in molecular guidance that is important for blastocyst attachment and subsequent invasion.

Kruppel-like factor 5 (*Klf5*), a zinc finger–containing transcription factor, is also not directly regulated by ovarian hormones in the uterus but is critical for implantation.[213] In mouse uteri, KLF5 is expressed in the luminal and glandular epithelia but disappears with initiation of decidualization on day 5. At this time, proliferating stromal cells around the implantation site show *Klf5* expression with simultaneous downregulation in the epithelium. Decidual cell expression disappears upon stromal differentiation. These results suggest that KLF5 participates in cell-specific proliferation and differentiation. Mice with uterine deletion of *Klf5* (*Klf5*$^{d/d}$) are infertile due to defective implantation.[213] Surprisingly, blastocysts remain entrapped within the uterine lumen past the anticipated time of implantation and can still initiate decidualization, albeit at a much reduced extent. *Hoxa10* and *Bmp2* expression in the stroma is crucial for decidualization; however, their expression is negatively impacted by *Klf5* deletion. This partial decidual response is not sustained, and embryos ultimately degenerate. These findings support an earlier study showing that a functional luminal epithelium is critical for the full-fledged decidual response.[214] Taken together, the results suggest that signals originating from a blastocyst yet to be defined are transmitted through the epithelium to initiate decidualization without direct physical contact of the blastocyst with the stroma.

Molecular Signature for Attachment Reaction and Implantation

Cross-talk between the implantation-competent blastocyst and receptive uterus is essential for the attachment reaction, an initial step in implantation. The attachment reaction coincides with increased uterine vascular permeability solely at the site of blastocyst apposition.[4,13] The differentiation of the uterus to the receptive stage and blastocyst attachment to the luminal epithelium are coordinated by diverse and overlapping gene expression patterns. The expression of various growth factors and their receptors in the uterus in a temporal and cell-specific manner during the peri-implantation period suggests that these factors are important for implantation.

The EGF family of growth factors includes EGF itself, transforming growth factor alpha (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, and neuregulins.[136,215] HB-EGF has emerged as an important molecular link in directing embryo–uterine interactions for the attachment reaction in mice. It is the earliest molecular marker to be found in the uterine luminal epithelium exclusively at the sites of blastocysts, appearing several hours before the attachment reaction in mice[9] (Figure 38.6(A)). This induction is followed by the expression of betacellulin, epiregulin, neuregulin 1, and COX2 around the time of the attachment reaction.[136,215,216] In contrast, amphiregulin is expressed throughout the uterine epithelium on the morning of day 4 of pregnancy and is well characterized as a P_4-responsive gene in the uterus.[217] Around the time of the attachment reaction, strong expression of amphiregulin in the luminal epithelium is only found around the implanting blastocysts, and this expression is absent by the morning of day 5. However, amphiregulin-deficient mice or compound knockout mice for EGF–TGFα–amphiregulin apparently do not exhibit implantation defects.[218,219] Since HB-EGF, betacellulin, epiregulin, neuregulin, and amphiregulin all show overlapping uterine expression patterns around the blastocyst at the time of attachment reaction,[74,215] it is possible that a compensatory mechanism rescues implantation in the absence of one or more members of the EGF family.

EGF-like growth factors interact with the receptor subtypes of the erbB gene family, which is composed of four receptor tyrosine kinases: ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4. They share common structural features, but differ in their ligand specificity and kinase activity.[221] The initial dimerization between coexpressed receptors upon ligand binding constitutes the classical mechanism

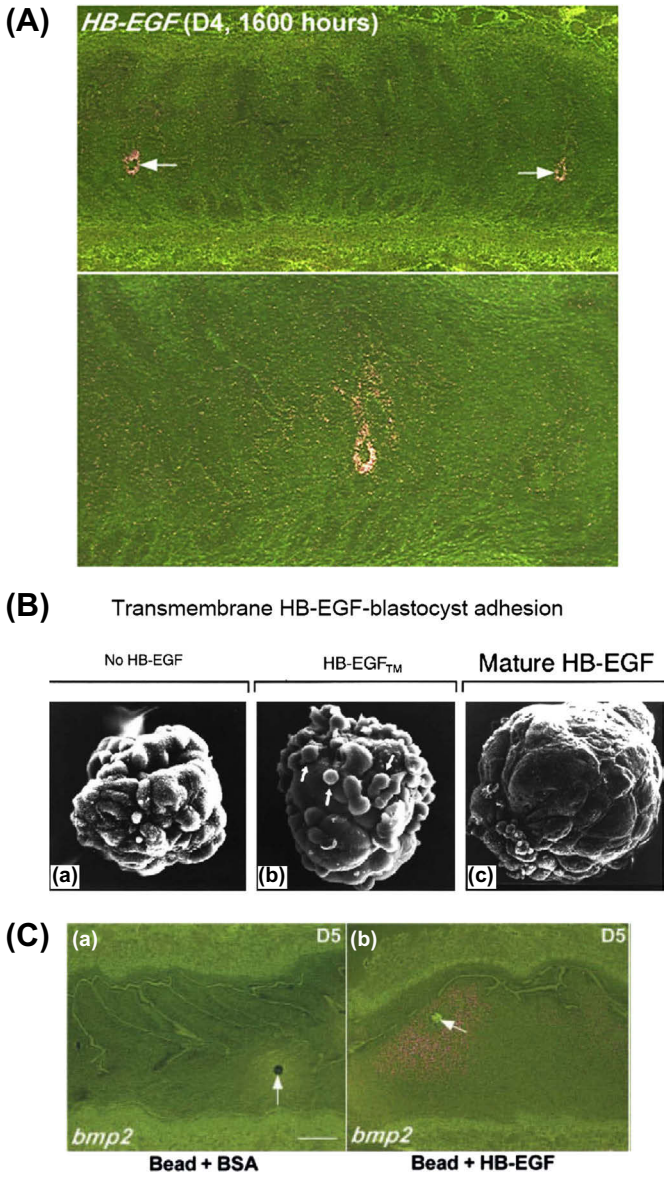

FIGURE 38.6 HB-EGF serves as a reciprocal mediator between the luminal epithelium and activated blastocyst during attachment reaction. This figure is reproduced in color in the color plate section. (A) In situ hybridization of HB-EGF mRNA (dark-field) in the mouse uterus at 1600 h on day 4 of pregnancy. Note distinct hybridization signals in luminal epithelial cells surrounding two blastocysts in a longitudinal section. Arrows demarcate location of blastocysts. (B) Scanning electron microscopy of blastocysts co-cultured with 32D cells (a murine myeloid cell line). Zona-free day 4 (0900 h) mouse blastocysts were co-cultured with (a) parental 32D cells, (b) 32D cells displaying transmembrane form of HB-EGF^{TM}, or (c) 32D cells synthesizing soluble form of HB-EGF for 36 h. After extensive washing to remove loosely adhering cells, blastocysts were fixed and examined by scanning electron microscopy; magnification 800×. Arrows point to 32D cells. (C) *Bmp2* gene expression in response to beads preloaded with HB-EGF. Beads (7 beads/horn) preabsorbed either in BSA (control) or HB-EGF (100 ng/ml) were transferred into uterine lumens of day 4 pseudopregnant mice. Mice were killed on day 5 to examine *Bmp2* expression at the sites of beads. Arrows indicate the locations of the beads. Note localized stromal expression of *Bmp2* at the sites of beads preabsorbed with HB-EGF. Bar: 75 mm. *Source: Reprinted with permission from Refs 9,135,220.*

of action of EGF-like ligands. Spatiotemporal expression patterns of EGF gene family members and ErbBs in the uterus during the peri-implantation period suggest compartmentalized functions of EGF-like growth factors in implantation.[136]

Numerous growth factors and their receptors are expressed in preimplantation embryos of several species, suggesting their roles in preimplantation mammalian development.[222,223] ErbB1 (EGFR), ErbB2, and ErbB4, the receptor subtypes for the EGF family of growth factors, are expressed in the mouse blastocyst,[123,224] and EGF or TGFα has beneficial effects on embryonic development in vitro.[225] There is evidence that embryonic ErbB1 and/or ErbB4 interact with uterine HB-EGF during blastocyst implantation, making HB-EGF a unique molecule for blastocyst–luminal epithelium adhesion.[135,224] HB-EGF is expressed in both soluble and transmembrane forms in the uterine luminal epithelium at the site of the blastocyst prior to the attachment reaction.[9,144] Both ErbB1 and ErbB4 are expressed in implantation-competent blastocysts, but downregulated in dormant blastocysts during delayed implantation.[123,224] Furthermore, while a recombinant soluble HB-EGF can promote blastocyst growth and differentiation, cells that express the transmembrane form of HB-EGF can adhere to active, but not dormant, blastocysts in vitro, suggesting HB-EGF's paracrine and juxtacrine effects[135] (Figure 38.6(B)). By directing an HB-EGF–toxin conjugate toward wild-type and erbB1$^{-/-}$ blastocysts, it was found that HB-EGF could also interact with embryonic ErbB4 and heparan sulfate proteoglycans.[224] Furthermore, Affi-Gel blue beads carrying recombinant HB-EGF can elicit implantation-like responses with Bmp2 induction and subsequent decidualization upon transfer to a receptive uterus (Figure 38.6(C)).[220] Collectively, these results suggest that an interaction between uterine HB-EGF and blastocyst ErbBs is important for the attachment reaction. However, the absolute necessity of HB-EGF in implantation requires additional genetic evidence. While global *Hegf1* deletion exhibits perinatal lethality,[226] its conditional deletion in the uterus defers blastocyst implantation beyond the normal window, producing smaller litter size; complete pregnancy failure is avoided at least in part due to compensatory actions by amphiregulin.[227] It should also be noted that early events of implantation do not appear to be affected by blastocysts deficient in either ErbB1 or ErbB4,[228,229] although the implantation-initiating efficiency of blastocysts deficient in more than one receptor type needs to be tested to delineate the functional redundancy among the receptor family.

Implantation-competent blastocysts can produce HB-EGF, and this growth factor induces its own expression in the uterus in a paracrine manner.[133] The results elucidate an important dual role of HB-EGF in both the blastocyst and uterus during implantation. These results provide

evidence that one of the signaling molecules involved in establishing a hierarchy of events between the embryo and uterus for implantation is HB-EGF, which originates in implantation-competent blastocysts. This autoinduction loop of HB-EGF is perhaps the first example of molecular cross-talk between these two different entities that leads to the initiation of the implantation process. In conclusion, detailed expression and gene-targeting experiments with all of the EGF family of ligands and receptors will be required to define the paracrine, autocrine, and/or juxtacrine roles of a specific ligand or its receptors in implantation.

These studies in mice led to investigation of the function of HB-EGF in human implantation. Among many growth factors, HB-EGF appears to play a role in implantation and embryonic development in humans. Its expression is maximal during the late secretory phase (cycle days 20–24) when the endometrium becomes receptive for implantation[230,231] and is co-expressed with pinopodes.[232] In addition, cells expressing the transmembrane form of HB-EGF adhere to human blastocysts displaying cell surface ErbB4.[233] In fact, HB-EGF was shown to be one of the most potent growth factors for enhancing the development of human IVF-derived embryos to blastocysts and subsequent zona hatching.[234] Thus, cumulative evidence suggests that HB-EGF has a significant role in preimplantation embryo development and implantation as a paracrine and/or juxtacrine factor in various species.

Temporal and cell-specific expression of TGFβ and receptor isoforms in the uterus during the peri-implantation period also suggests that this growth factor is also important for implantation.[235–237] TGFβ was shown to be involved in the implantation process by modulating immunological responses. TGFβ derived from the seminal vesicle gland was identified as a major active constituent in the seminal plasma of several mammalian species, where it reaches concentrations >200 ng/ml.[238] In mice, exposure to semen at mating activates an inflammatory response in the uterine mucosa,[239] and in women, sexual intercourse causes similar inflammatory events in the cervix.[240,241] The response is activated when seminal TGFβ triggers synthesis of several pro-inflammatory cytokines and chemokines in female reproductive tract epithelial cells, notably granulocyte macrophage colony-stimulating factor (GM-CSF) and IL6.[242]

Many glycoproteins and carbohydrate ligands and their receptors are expressed in the uterine luminal epithelium and blastocyst cell surfaces around the time of implantation.[243,244] Primary adhesion molecules that are implicated in implantation are selectins, mucin 1 (Muc1), integrins, and the trophinin–tastin–bystin complex. The selectin adhesion system likely plays an important role in human implantation.[245] On the maternal side, selectin oligosaccharide ligands are expressed in the receptive uterine epithelium, and on the embryonic side trophoblast cells express L-selectin receptors. Beads coated with specific selectin ligands adhere to the trophoblast, suggesting that the trophoblast cell surface receptors are functional. This study suggests that trophoblast L-selectin mediates interactions with the uterus to establish an adhesion mechanism for implantation.

Muc1, a stretch of long carbohydrate moieties, is expressed on the apical surface of the mouse luminal epithelium during the prereceptive period. Muc1 acts as an antiadhesive masking molecule. The physical hindrance created by these carbohydrate branches is considered to prevent interaction between the embryo and the luminal epithelium of the uterus prior to the attachment reaction.[246] This is consistent with timely downregulation of Muc1 from the luminal epithelium throughout the uterus before the attachment reaction on day 4 of pregnancy in mice.[74] This seems paradoxical to the observation that overall Muc1 expression increases in the rabbit and human uterus during the receptive period. However, it was later revealed that there is indeed a decrease in Muc1 levels at the site of implantation in rabbits.[247] In humans, the situation appears to be more complicated. During the apposition phase, the presence of an embryo increases the levels of Muc1 in the epithelium, but at the adhesion phase, the embryo induces cleavage of Muc1 to clear this glycoprotein from the implantation site.[248] Collectively, these findings suggest that Muc1 acts as an antiadhesive molecule that must be removed from the implantation site.

Extracellular matrix (ECM) and integrins are thought to be responsible for trophectoderm attachment and adhesion to the luminal epithelium. Each integrin is composed of two subunits, α and β, and each αβ combination has its own binding specificity and signaling properties. As membrane-associated receptors, integrins possess short cytoplasmic tails with no enzymatic activity. Signaling by integrins is mediated by associating adaptor proteins that bridge them to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors.[249] Integrin subunits αv, α4, α5, β1, β3, and β5 are constitutively expressed on both the conceptus trophectoderm and the apical surface of the luminal epithelium in ewes during the peri-implantation period.[88] The members of the integrin family serve as receptors for various ECM ligands and modulate cell–cell adhesion and signal transduction cascades.[249] Trophoblast interactions with the ECM are mediated primarily by integrins,[250–256] and several ECM components that are upregulated in the peri-implantation endometrium, including fibronectin, laminin, and collagen type IV,[257–259] support trophoblast outgrowth in vitro.[254,256] Several members of the integrin family including αvβ3 are known to interact with the RGD (Arg–Gly–Asp) peptide sequence present in many ECM proteins, such as fibronectin, collagen type II and

IV, entactin, and vitronectin[254–256]; hexapeptides containing the RGD sequence can block trophoblast outgrowth on many ECM proteins.[260] Notably, trophoblast adhesion to type I laminin is independent of its RGD sequence and is primarily mediated through interaction of α7β1 with the E8 integrin recognition domain of laminin.[253,261]

Integrins are expressed in the human uterus with both constitutive and cycle-dependent patterns of expression.[262,263] Characterization of integrin expression during the menstrual cycle further defined three specific integrin family members expressed during a narrowly defined time between days 20 and 24 of a typical 28-day cycle in women.[264–268] Among the integrins, αvβ3 has been shown to be localized to the luminal epithelium.[269] Temporal and spatial expression of this integrin and its ligand osteopontin (OPN) correspond to the specialized surface modifications known as pinopodes,[270,271] which are also expressed during implantation in other animal species.[272–274] In mice, αvβ3 is expressed in both the uterine luminal epithelium and the blastocyst during implantation. It has been shown that an intrauterine injection of an RGD peptide or a neutralizing antibody against αvβ3 reduces the number of implantation sites in mice and rabbits.[272] Lower expression of αvβ3 has been associated with infertility and recurrent pregnancy loss in women. In women with histologic delay (luteal phase defect) or other diagnoses associated with suspected implantation defects, integrin expression is consistently delayed or absent.[262,265,275–279]

Among others, α5β1, α6β1, and αvβ3 are expressed in the mouse embryo throughout the peri-implantation period, while several others exhibit stage-specific expression.[250] Integrins are also expressed in the differentiating trophoblasts at later stages,[250] suggesting their roles in trophoblast differentiation and adhesion. A role for fibronectin via integrin binding in blastocyst outgrowth was further confirmed in vitro using antibodies against αv, α5, β1, or β3 that are inducible by fibronectin inhibited adhesiveness of the outer surface of the trophoblast.[252] In addition, a gene-targeting experiment revealed that deletion of the β1 gene results in ICM defects and embryonic lethality.[280] However, the mutant embryos form morphologically normal blastocysts and initiate implantation, but trophoblast invasion becomes defective. Adhesion-competent, late-blastocyst-stage trophoblasts undergo intracellular signaling initiated upon ligation of α5β1 and αvβ3 by fibronectin.[281] Integrin signaling mobilizes cytoplasmic Ca^{2+} and induces the trafficking of intracellular vesicles, resulting in stronger adhesion to fibronectin at the apical surface. Therefore, blastocyst adhesion to the endometrium during implantation is considered to be regulated by the endogenous developmental program, as well as through interactions with ECM components in the local environment.[282,283] Although there is evidence that the embryo is a site of action for integrin signaling, it is not yet clear if the uterus is also a site of action of this signaling. Results of gene-targeting experiments of integrin subunits are not very informative in relation to their roles in implantation because of the complex phenotypes and apparent compensation by other subunits.[284–287]

Invasive mouse trophoblasts adhere, spread, and migrate on ECM substrates,[251,254,288] and penetrate three-dimensional ECM structures.[62,289] OPN is an acidic phosphorylated glycoprotein component of the ECM detected in the epithelium and in secretions of many tissues, including the uterus.[290] OPN binds to integrin heterodimers (αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α4β1, α5β1, and α8β1) via its RGD sequence and to α4β1 and α9β1 by other sequences to promote cell adhesion, spreading, and migration.[290] OPN is implicated in implantation in ruminants and humans. In sheep, OPN expression is increased in pregnant sheep uteri during the peri-implantation period (days 11–17) when attachment of concepti to uterine luminal epithelium occurs.[291,292] Secreted OPN binds to integrin heterodimers expressed by the trophectoderm and the uterus to (1) stimulate changes in morphology of the conceptus extraembryonic placental membranes, and (2) induce adhesion between the luminal epithelium and trophectoderm for implantation and placentation.[290] OPN is also present in uterine luminal flushings from cyclic and pregnant gilts.[293] In humans, the spatiotemporal distribution of OPN protein correlates with the expression pattern of αvβ3 integrin, appearing approximately 7 days after ovulation.[294] In the mouse uterus, OPN is highly expressed in the metrial gland in the developing decidua.[295] However, OPN-deficient mice are fertile, suggesting OPN is not essential for fertility in mice.[296]

Trophinin is a transmembrane protein that can mediate homophilic interactions between two different cell types, including an interaction between human endometrial and trophoblastic cell lines.[297] Trophinin requires the presence of a cytoplasmic protein, tastin, to sustain adhesion between these two cell types. In addition, the presence of bystin, another cytoplasmic protein, is required for effective interaction between trophinin and tastin. This adhesion complex that is present in trophoblastic teratocarcinomas and endometrial adenocarcinomas mediates adhesion between them. In humans and monkeys, trophinin is specifically expressed in cells involved in implantation. Furthermore, the trophinin complex was detected in trophoblast and decidual cells at the human fetal–maternal interface as early as the sixth week of pregnancy.[298] Trophinin, tastin, and bystin are all highly expressed in fallopian tube epithelial cells of women with tubal pregnancies, suggesting their role in ectopic pregnancy, a condition unique to humans.[299,300] In mice, trophinin expression is distinct from that in humans and trophinin-deficient mice do

not exhibit fertility defects, suggesting that this adhesion molecule is not crucial to implantation and placentation in mice.[300] However, trophoblast cell activation by trophinin ligation has been shown in human trophoblast (HT-H) cells[301] with apoptosis of human endometrial epithelial cells via PKCδ signaling.[302]

Decidualization

Following blastocyst attachment with the luminal epithelium, decidualization is initiated at the antimesometrial site where blastocysts implant. This process, characterized by stromal cell proliferation and differentiation into a specialized type of cells (decidual cells) with polyploidy, is critical to the establishment of pregnancy in many species. Normally, the implanting blastocyst is the stimulus for decidualization. However, a similar process (deciduoma) can be experimentally induced in the pseudopregnant or hormonally prepared rodent uterus by intraluminal infusion of various agents, including oil.[13] In 1908, Leo Loeb was the first to induce tumorlike deciduoma in guinea pigs and described that this event required an endometrium, hormonal conditioning, and stimulation by a nonspecific stimulus such as glass beads.[303,304] It was later found that other nonspecific stimuli such as intraluminal infusion of oil, air, or trauma can also initiate deciduoma in pseudopregnant or steroid hormonally prepared uteri.[13] This field was later advanced by the work of M.C. Shelesnyak, Vincent Defeo, Bruce Moulton, J.M. Yochim, and others.[305–308] However, there is evidence that the initial uterine reactions induced by nonspecific stimuli are different from those induced by blastocysts.[220,309]

The decidual cell reaction is always preceded by increased endometrial vascular permeability.[4,310] Pulse-labeling experiments with ^{3}H-thymidine provided evidence that decidual cells originate from undifferentiated stromal cells.[307,311] In mice, differentiating stromal cells surrounding the blastocyst initially form the primary decidual zone (PDZ) on day 5. This zone is avascular and densely packed with epithelioid-type cells. By day 6, the PDZ is well formed, and a secondary decidual zone (SDZ) is formed at the periphery of the PDZ, which degenerates progressively up to day 8. At this time, DNA synthesis is high in the SDZ, but very low in the PDZ.[35] After day 8, placental and embryonic growth slowly replaces the SDZ, which is reduced to a thin layer of cells called the decidua capsularis. The mesometrial decidual cells ultimately form the decidua basalis.[312] Presumed functions of decidua are to provide nutrition to the developing embryo, protect the embryo from immunological responses of the mother, regulate orderly trophoblast invasion into the uterine stroma, and direct placentation.

Decidualization involves a complex interplay of cell cycle regulators, transcription factors, morphogens, cytokines, and signaling pathways. The mechanisms by which the cell cycle events govern decidualization are poorly understood. The cell cycle is tightly controlled at two checkpoints: the G1-S and G2-M phases. Normally, the operation of these phases involves a complex interplay of cyclins, cyclin-dependent kinases (cdks), and cdk inhibitors (CKIs). The well-known regulators of mammalian cell proliferation are the three D-type cyclins (D1, D2, and D3).[313] In mice, the expression of cyclin D3 is upregulated in decidualizing stromal cells at the implantation site and is associated with cell proliferation.[314–316] Furthermore, cyclin D3 is associated with the large polyploid cells that are defined as terminally differentiated stromal cells. Coordinated expression of cdk4 and cyclin D3 at the site of implantation in mice on day 5 suggests that these regulators play roles in decidualization. However, the expression of p21 with concomitant downregulation of cyclin D3 and cdk4 in the PDZ at the implantation site on the afternoon of day 5 supports the view that cell proliferation activity of cdk4–cyclin D3 ceases with the development of the PDZ. On the other hand, the expression of cdk4–cyclin D3 in the decidualizing stroma outside the PDZ is consistent with their role in proliferation of the stroma at the SDZ. The presence of cyclin E, cyclin A, and cdk2 with concomitant downregulation of cyclin B and cdk1 in these cells supports the view that these cells are entering the endocycle pathway. The physiological significance of stromal cell polyploidy during decidualization is still unclear. The life span of decidual cells during pregnancy is limited, and their demise makes room for the rapidly growing embryo. Since most decidual cells become polyploid during their lifetime, it is speculated that polyploidy limits the life span of decidual cells. Furthermore, one of many functions of the deciduum is to support embryonic growth that requires increased protein synthesis. Polyploidy thus may ensure increased synthetic capacity by increasing the number of gene copies for transcription.

Uterine luminal epithelial competency is critical for normal decidualization, suggesting that signals emanating from the epithelium influence stromal decidualization.[214] This is now evident from mouse studies in which luminal epithelial function was compromised.[151,213] For example, *Ptgs2* is normally expressed in the epithelium and stroma at the site of the implanting blastocyst.[317] In systemic *Lif*$^{-/-}$ or females with uterine deletion of *Msx1/Msx2*, *Ptgs2* expression is restricted to the luminal epithelium,[151,176] whereas *Ptgs2* is expressed only in the stroma in females with uterine deletion of *Klf5* around the time of implantation.[213] These results suggest aberrant communication between the luminal epithelium and stroma. In conclusion, a functional loop involving the blastocyst, luminal epithelium, and stroma appears critical to proper decidualization. Further studies are

needed to identify the blastocyst- and/or epithelial-derived signals critical to decidualization.

In mice, abdominalB (*AbdB*)-like homeobox (*Hox*) genes *Hoxa10* and *Hoxa11* are expressed in the stroma of the receptive uterus with robust increases upon decidualization, and are essential for decidualization. *Hoxa10*$^{-/-}$ females have decidualization failure due to reduced P_4 responsiveness and dysregulation of P_4-responsive genes with reduced stromal cell proliferation.[318,319] *Hoxa11*$^{-/-}$ females have a similar but more intense infertility phenotype.[320] The defective decidualization in *Hoxa10*$^{-/-}$ mice is also reflected in downregulation of a cell cycle regulatory axis engaging cyclin D3, cdk6, and p21.[318,320] In women, *Hoxa10* and *Hoxa11* are upregulated in the receptive endometrium, suggesting their importance in decidualization.[137,321] SGK1 (serum- and glucocorticoid-inducible kinase 1), a regulator of epithelial ion transport and cell survival, is reported to be critical for implantation and maintenance of the decidual–placental interface integrity in mice. SGK1 also showed differential expression patterns in pregnancy pathologies in women.[322] Mice with deletion of cytokine receptor *IL11Rα1* or sphingosine kinases *Sphk1/Sphk2* manifest aberrant decidualization as well.[3,323,324]

In vitro culture systems modeling human decidualization induced by P_4, estrogen, and dibutyryl cAMP involve numerous signaling molecules, including prolactin and insulin-like growth factors (IGFs). In humans, decidualizing stromal cells express high levels of IGF binding protein 1 (IGFBP1).[325–328] Its proposed roles in reproductive physiology are numerous, and its aberrations are implicated in preeclampsia, fetal growth retardation, and polycystic ovarian syndrome.[328] Similarly demonstrated in the liver system, IGFBP1 expression is regulated in primate uterine stromal cells by binding of the transcription factors HOXA10 and FKHR (Forkhead box protein O1, also called FOXO1) to its promoter.[329]

A large number of decidual cells undergo endo-reduplication (polyploidy) (i.e., repeated rounds of DNA replication without cytokinesis). Decidual endo-reduplication is considered to support embryonic growth by increasing gene transcription. Decidual cell polyploidy is critical to pregnancy success in mice, since mice with inactivation of death effector domain-containing protein (DEDD) show faulty decidualization with reduced polyploidy and embryonic loss prior to placentation, leading to infertility; the attachment reaction is normal.[330] DEDD forms a complex with cyclin D3, cdk4/6, and protein kinase B to execute its function. While decidual polyploidy is well established in rodents, this phenomenon is only sparsely reported in humans[331] and requires closer assessment.

As mentioned here, stromal cells at the blastocyst attachment site undergo decidualization. The initiation of this process ("predecidualization") in humans can occur during receptivity in the absence or presence of a blastocyst, but becomes more robust with implantation. The significance of "predecidualization" in humans may be to prepare the endometrium for implantation and seems analogous to heightened stromal cell proliferation with expression of decidual marker genes prior to implantation in mice.

Decidualization is a critical step toward appropriate placental development. The nature of placentation varies between species and is broadly classified according to the degree of invasiveness and association of maternal and fetal structures. Placentation strategies range from simple superficial epitheliochorial placentas of domestic animals (horses, cows, sheep, and pigs) to the endotheliochorial placentas in carnivores and hemochorial placentas in rodents, humans, and nonhuman primates. In the epitheliochorial type of placentas, the uterine luminal epithelium remains intact along with the embryo, and associated membranes are restricted to the uterine lumen throughout gestation; this is as opposed to endotheliochorial and hemochorial types of placentas, in which the conceptus invades and/or erodes the uterine epithelium and embeds into the endometrial stroma.[332] Despite the diversity of placentation strategies, the initial events of apposition, attachment, and adhesion between the uterine luminal epithelium and conceptus trophectoderm are shared among species. In sheep, the trophectoderm adhesion to the epithelium progresses along the uterine horn and appears to be completed around day 22.[21,24] Unlike sheep, the attachment in horses and pigs involves multiple sites covering most of the embryonic surface. In all of these ruminants, a notable decidualization is absent.

Signaling Spanning Different Phases of Early Pregnancy

It is difficult to understand the stage-specific functions of many critical genes due to their overlapping uterine expression at more than one stage of pregnancy. One such gene is *Lif*, which is expressed in a biphasic manner in the uterus, first in glands on the morning of day 4 and then in the subepithelial stroma at the site of blastocyst attachment on the evening of day 4[333]; to date, it has not been clarified which phase of *Lif* expression is essential for implantation. A notable example is *Pla2g4a*$^{-/-}$ mice, which lack the enzyme cytosolic phospholipase A2α (cPLA2α). Mice with this deletion exhibit aberrant implantation with retention of glandular *Lif* expression but a loss of stromal *Lif* expression.[47] Cell-type-specific deletion may help dissect the contribution of each phase of *Lif* expression. Other such molecules are Hand2, KLF5, BMP2, and COX2, which are expressed at the time of blastocyst attachment and persist during decidualization.

Prostaglandins

Since implantation is thought to be a proinflammatory reaction, one early detectable marker is an increased uterine vascular permeability at the site of blastocyst attachment. COX-derived PGs have been shown to facilitate these effects.[317] PGs possess vasoactive, mitogenic, and differentiating properties[334] and are implicated in various female reproductive functions. Early studies showed that during artificially induced decidualization, uterine concentrations of PGs increase in a temporal pattern accompanied by increases in endometrial vascular permeability, an early event in the endometrial response to decidualization.[335–340] These studies used pharmacological inhibitors of PG synthesis, which substantially attenuated the increased endometrial vascular permeability changes[335,341,342] and subsequent decidualization.[338,343,344]

PGs are produced via the COX pathway. COX exists in two isoforms, COX1 and COX2, and is the rate-limiting enzyme in the biosynthesis of PGs. COX mediates the conversion of arachidonic acid into PGH_2, which is then converted to various PGs by specific synthases.[334] The COX isoforms are encoded by two separate genes and exhibit distinct cell-specific expression, regulation, and subcellular localization, although they share similar structural and kinetic properties. COX1 is considered a constitutive enzyme that mediates "housekeeping" functions. In contrast, COX2 is an inducible enzyme and is induced in a variety of cell types by growth factors, cytokines, and inflammatory stimuli.[334] Since COX2 is primarily responsible for increased PG production during inflammation, this isoform is the target for developing selective anti-inflammatory drugs.[345,346] COX2 overexpression is also associated with tumorigenesis.[347,348] PGs normally execute their functions by interacting with cell surface G protein–coupled receptors, but they can also function as ligands for nuclear peroxisome proliferator-activated receptors (PPARs).[349–352]

Because of the "proinflammatory" characteristics of ovulation and implantation, participation of PGs in these processes has been speculated.[353,354] For example, PGs are considered to participate in follicular rupture during ovulation[355] (see Chapters 22 and 28). This is consistent with gonadotropin-mediated induction of PG endoperoxide synthase 2 (*Ptgs2*, encoding Cox2) in ovarian follicles preceding ovulation.[355,356] PGs are also implicated as important mediators of increased endometrial vascular permeability during implantation and decidualization.[216] Both *Ptgs1* and *Ptgs2* exhibit a unique pattern of expression in the peri-implantation mouse uterus.[216] *Ptgs1* is expressed in uterine luminal and glandular epithelial cells on the morning of day 4 of pregnancy, but its expression is downregulated in the luminal epithelium by the time of the attachment reaction. This ubiquitous expression suggests its role in generalized uterine edema that may be involved in luminal closure for blastocyst apposition. In contrast, *Ptgs2* is expressed in the luminal epithelium and underlying stromal cells at the antimesometrial pole solely at the site of blastocyst attachment. *Ptgs2* expression then moves to the mesometrial side (the presumptive site of placentation) by day 6 of pregnancy.[317] Employing the delayed implantation model, this study also showed that the expression of *Ptgs2* in the receptive uterus requires the presence of active blastocysts. Gene-targeting experiments further demonstrated that COX2-derived PGs are essential for ovulation, fertilization, implantation, and decidualization, asserting that COX2-derived PG signaling is required at several stages of pregnancy.[317,357,358] Experiments with $Ptgs1^{-/-}$ mice suggest that the loss of COX1 is compensated by the expression of COX2 for implantation.[359] Among various PGs, the levels of prostacyclin (PGI_2) are highest at the implantation sites of wild-type mice and implantation defects are partially restored in $Ptgs2^{-/-}$ mice by administration of a more stable prostacyclin agonist, carbaprostacyclin.[360] A recent study reports that the activation of epithelial sodium channels can induce *Ptgs2* for implantation.[361]

The role of PGs is further illustrated by the reduced fertility of female mice lacking $cPLA_2\alpha$, which is involved in the liberation of arachidonic acid from membrane phospholipids for PG synthesis by the COX system.[362–364] The reduced fertility in these females is due to deferral of "on-time" implantation, leading to subsequent retarded feto-placental development and reduced litter size.[47] Collectively, these results indicate that the $cPLA_2$–COX2 axis is crucial to implantation.

COX2 is also expressed in the uterus, blastocyst, or both during implantation in a variety of species with different modes of implantation, including sheep, minks, skunks, baboons, pigs, and hamsters.[148,149,212,365,366] In hamsters, COX2 and PGE synthase are coexpressed and produce PGE_2 as a major PG product in implantation sites.[212] COX2 expression in human endometrium has also been reported.[367,368] These results suggest a conserved function of COX2 in implantation in various species. It has also been shown that, depending on the genetic background, *Ptgs1* upregulation can improve female infertility in *Ptgs2*-deficient mice.[369]

Membrane receptors for PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxanes are prostaglandin E (EP_1–EP_4), prostaglandin F (FP), prostaglandin D (DP), prostaglandin I (IP), and thromboxane A (TP), respectively; they belong to the G protein–coupled family of cell surface receptors.[370,371] Although PGE_2 synthase is expressed at the implantation sites with the presence of PGE_2 and EP receptors,[371–373] and although PGE_2 has been shown to be associated with implantation and decidualization,[338] gene-targeting experiments show that three of the four EP receptor subtypes (EP_1–EP_3) are not critical

to implantation. EP_4 deficiency mostly results in perinatal lethality, and thus its role in implantation has not yet been fully explored.[371] Mice deficient in FP or IP show normal implantation. PGs can also exert their effects by utilizing PPARs that belong to a nuclear hormone receptor superfamily.

PGs also appear to be important for embryonic functions relevant to preimplantation embryo development and implantation. Preimplantation embryos produce PGs, and inhibitors of PG synthesis have been shown to inhibit embryonic growth, functions, and zona hatching in vitro.[245,374] Dormant mouse blastocysts can achieve implantation competence if cultured in the presence of PGE_2 or a permeable analog of cAMP. This effect apparently involves the COX2-signaling pathway,[67] yet normal development of *Ptgs1*$^{-/-}$/*Ptgs2*$^{-/-}$ double-mutant embryos in the uterus suggests that PGs of embryonic origin are not essential for embryo development.[375] However, compensation by maternal PGs in embryonic development cannot be ruled out.

The nuclear receptor superfamily of transcription factors modulates expression of target genes by binding to specific DNA elements. The members of this superfamily span from well-characterized steroid hormone receptors to orphan nuclear receptors with no known ligands. Steroid hormone receptor aside, the PPAR family of nuclear receptors has been implicated in female reproductive events. Three members of the PPAR family are PPARα, PPARγ, and PPARδ. To act as a transcriptional activator, PPARs must heterodimerize with a member of the retinoid X receptor (RXR) subfamily.[351,360] The ligands for PPAR include natural and synthetic eicosanoids, fatty acids, and hypolipidemic and hypoglycemic drugs, and there is evidence that PPAR–RXR dimers mediate nuclear signaling of PGs.[376]

PPARδ has been shown to participate in various physiological processes, including embryo implantation.[360] As mentioned here, PGs produced via the COX2 pathway are essential for implantation. During early pregnancy in mice, PGI_2 is the most abundant PG in the uterus, and its levels are higher at implantation sites than at interimplantation sites. Consistent with the finding that COX2-driven uterine PG production is crucial to implantation, *Ptgs2* and PGI synthase (*PGIS*) are coexpressed at implantation sites, suggesting the availability of PGI_2 to uterine cells. Among known PGI_2 receptors, such as IP, PPARα, and PPARδ, PPARδ is co-localized with *Ptgs2* and *PGIS* at similar regions of the implantation sites; the expression of IP and PPARα was very low to undetectable. PPARδ expression requires the presence of active blastocysts, since it is not detectable in the uteri of delayed-implanting mice.[377]

PPARδ is indeed functional as a PGI_2 receptor, since administration of cPGI or L-165,041 (a selective PPARδ agonist) to *Ptgs2*-deficient mice improves implantation and decidualization,[360] providing evidence for a role for PPARδ in embryo implantation. Three independent groups have reported diverse phenotypes of PPARδ knockout mice.[378–380] Because of severe early developmental defects of PPARδ mutant embryos, it has been very difficult to utilize this model to directly address whether the absence of maternal PPARδ affects implantation as in *Ptgs2*-deficient mice. While maternal PPARδ is considered critical for implantation and decidualization, embryonic PPARδ is necessary for placentation.[381] Therefore, a mouse model with stage- and uterine-specific deletion of PPARδ is necessary to address this issue. Uterine expression of PPARδ has also been reported in rats and humans.[382,383]

Endocannabinoid Signaling

Psychoactive cannabinoids are active components of marijuana that work via activation of the G protein–coupled cell surface receptors, CB1 and CB2, encoded by *Cnr1* and *Cnr2*, respectively.[384,385] The discovery of these receptors led to the identification of endogenous cannabinoid ligands (endocannabinoids), arachidonoylethanolamine (anandamide, also abbreviated as AEA) and 2-arachidonoylglycerol (2AG).[386,387] The mouse uterus synthesizes anandamide, and the fluctuation of its uterine level during early pregnancy coincides with the window of uterine receptivity for implantation.[388] Indeed, this signaling pathway plays significant roles in embryo development, oviductal embryo transport, uterine receptivity, placentation, and parturition in mice,[389] asserting that the endocannabinoid system is active at most stages of pregnancy. Anandamide levels were found to be lower in the receptive uterus and at the implantation sites, but were higher in the nonreceptive uterus and at interimplantation sites.[388] The P_4-treated delayed implantation uterus also showed elevated levels of anandamide, but the levels were downregulated with the termination of delayed implantation by estrogen.[138] Ligand-receptor signaling with cannabinoids and its receptor CB1 also directs preimplantation embryo development and implantation. CB1 is expressed in the embryo from the two-cell stage at the time of zygotic gene expression through the blastocyst stage.[390] Embryonic CB1 is functional, since two-cell embryos cultured in the presence of natural cannabinoids, synthetic cannabinoids, and endocannabinoids fail to develop to the blastocyst stage; this failure occurs between the eight-cell and blastocyst stages. The effect is reversible by a CB1-selective antagonist.[391] Furthermore, the endocannabinoid anandamide at a low concentration stimulates blastocyst differentiation and trophoblast outgrowth, while at higher concentrations it inhibits these events via differential regulation of MAPK and Ca^{++} signaling.[392,393] These results suggest that a narrow range of cannabinoid concentrations regulate the embryonic developmental program.

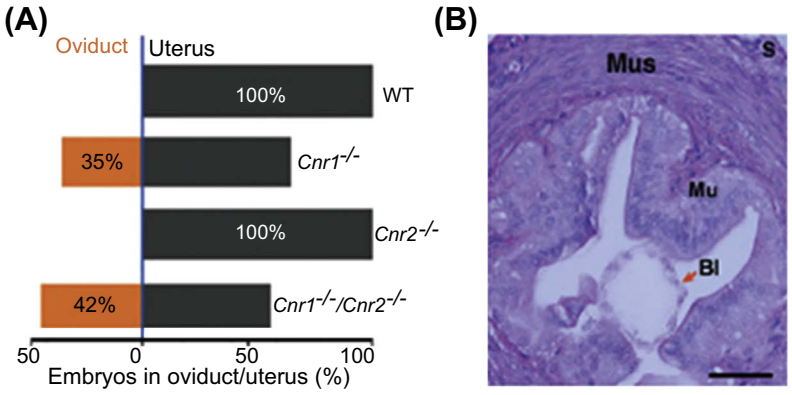

FIGURE 38.7 Impaired oviductal embryo transport causes pregnancy loss in *Cnr1*$^{-/-}$ but not *Cnr2*$^{-/-}$ mice. This figure is reproduced in color in the color plate section. (A) Percentage of embryos recovered from oviducts or uteri in WT and *Cnr1*$^{-/-}$ mice on day four of pregnancy. (B) A representative histological section of a day 7 pregnant *Cnr1*$^{-/-}$ oviduct showing an entrapped blastocyst (Bl, arrow) at the isthmus. Mus, muscularis; S, serosa; Mu, mucosa. Bar, 100 μm. *Source: Reprinted with permission from Ref. 394.*

A tightly regulated level of uterine anandamide and embryonic CB1 during early pregnancy is important for preimplantation embryonic development and implantation. Indeed, embryos develop asynchronously in CB1 (*Cnr1*) mutant mice during the preimplantation period. *Cnr1*$^{-/-}$ females show compromised fertility with blastocysts retention in the oviduct (Figure 38.7).[394] This retention is due to a collaboration of oviductal CB1 with adrenergic receptors to direct the timely passage of embryos through the oviduct. This is consistent with the later finding that Fallopian tubes of women with ectopic pregnancy show downregulation of *CNR1*.[395] Moreover, implantation fails to occur in wild-type mice, but not in *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ double-mutant mice, when they are maintained by experimentally induced, sustained levels of exogenously administered cannabinoids.[138] Notably, persistent high levels of AEA resulting from deficiency of *fatty acid amide hydrolase* (*Faah*, encoding FAAH) that metabolizes AEA leads to defective implantation.[396]

The observation that heightened levels of cannabinoids inhibit implantation in mice subsequently led to the discovery that elevated levels of anandamide due to its reduced metabolism induce spontaneous pregnancy losses in women.[397,398] Thus, regulated cannabinoid signaling perhaps functions as a physiological surveillance system that assures implantation of healthy embryos, but not of abnormal embryos that result from aberrant expression of CB1 or their exposure to aberrant levels of endogenous or exogenous cannabinoid ligands. Endocannabinoid signaling is also important for directing the differentiation of trophoblast stem cells, and its aberrant signaling gives rise to compromised placentation and trophoblast invasion in the mouse uterus.[399] Microarray analysis on embryos in which endocannabinoid signaling via CB1 is silenced (*Cnr1*$^{-/-}$) or elevated (*Faah*$^{-/-}$) leads to similar changes in downstream targets, compromising trophoblast migration.[400] Taken together, these results suggest that tight regulation of endocannabinoid signaling is critical for early pregnancy success.

Morphogens

The reciprocal communication between the uterus and embryo in implantation shares many features with epithelial–mesenchymal interactions during development, and both processes involve evolutionarily conserved signaling molecules, including BMPs, Wnts, and hedgehogs. In mice, *Bmp2* is locally expressed in the subepithelial stroma at the site of blastocyst attachment, followed by heightened expression in decidua.[220] Although BMP regulators Crim1 and Dan are expressed along with Bmp2 at later times, its antagonist Noggin is expressed in the stroma at the time of uterine receptivity but disappears with stromal *Bmp2* expression upon attachment and thereafter. Deletion of *Bmp2* in the uterus results in failure of decidualization and infertility with apparently normal attachment reaction.[401] Notably, Affi-Gel blue beads carrying recombinant BMP2 did not elicit implantation-like responses upon transfer to a receptive uterus, but they affected embryo spacing when blastocysts were co-transferred with the beads.[220] However, how BMP2 signaling influences decidualization in the context of BMP receptors and antagonists has not been explored in depth. Of the *Bmp* genes that have been examined in the receptive uterus, *Bmp4–Bmp7* and *Bmp8a* did not exhibit the highly localized expression pattern as seen for *Bmp2* during attachment. *Bmp7* is expressed during early decidualization; however, its function has yet to be determined.[220]

Spatiotemporal expression of Wnt ligands, receptors, and inhibitors in mouse and human uteri during the reproductive cycle and pregnancy implicates roles for Wnt signaling in early pregnancy events.[204,402,403] Wnt signaling is mediated by canonical and noncanonical signaling pathways with respect to subcellular localization of β-catenin, transcriptional regulation of target genes, and co-receptor identity. Studies in TOPGAL (a Wnt-signaling activity reporter) mice found canonical Wnt signaling in the luminal epithelium and myometrium at the time of blastocyst attachment, suggesting a role for canonical signaling in implantation.[404]

Wnt4 is expressed in the subepithelial stroma during implantation and becomes more intense in decidualization,[405] while its antagonist *sfrp4* is expressed in the uterine stroma during the receptive phase and is downregulated upon attachment.[204] Wnt4 expression is dysregulated in *Lif*$^{-/-}$ and *Hoxa10*$^{-/-}$ mice[204]; uterine deletion of Wnt4 confers infertility with defective adenogenesis.[405] In contrast, *Wnt7a* is expressed in the epithelium, and its systemic deletion incurs infertility with defective implantation and aberrant development of the oviduct, uterus, cervix, and vagina along the anteroposterior axis and is associated with loss of *Hoxa10* or *Hoxa11* with disorganized myometria.[406] Conditional uterine deletion incurs infertility and defective gland formation.[407] On the contrary, β-catenin overexpression leads to uterine glandular hyperplasia, suggesting a role for canonical signaling in gland formation.[408] Collectively, compromised fertility and implantation failure observed with uterine deletion of *Wnt4* and *Wnt7a*, respectively, are perhaps consequential to structural aberrations and sparse gland formation.

Genes encoding the components of the hedgehog-signaling pathway (i.e., Ihh, the multipass transmembrane hedgehog-binding protein/receptor, PATCHED (PTC), and the transcription factors GLI1–GLI3[409–411]) are expressed in a dynamic temporal and spatial pattern during preparation of the uterus for implantation.[194] The expression of Ihh increases in the luminal epithelium and glands starting from day 3 and reaches high levels on day 4. During the same time, the expression of Ptc, Gli1, and Gli2 is upregulated in the underlying mesenchymal stroma. Transcription of Ihh in ovariectomized mice is induced by P_4 but not by estrogen. Lower induction of Ihh, Ptc, and Hoxa10 is also observed in response to P_4 treatment in the uteri of PR mutant mice lacking nuclear PR. This finding suggests that this hormone regulates Ihh via both nuclear receptor–dependent and independent pathways. Furthermore, in uterine explant cultures, recombinant N-terminal mature fragment Sonic hedgehog stimulates the proliferation of mesenchymal cells and the expression of noggin. These findings suggest that hedgehog generated by the epithelium functions as a paracrine growth factor for stromal cells during the early stages of pregnancy.[194] P_4 regulation of Ihh expression in the mouse uterus was confirmed by another group of investigators.[412] Uterine deletion of Ihh shows implantation failure due to compromised uterine receptivity.[195]

Connexins

Connexins (Cx), the gap junction–forming proteins, direct intercellular communication and permit the passage of small molecules between the cytoplasm of neighboring cells, thereby coupling cells electrically and metabolically.[413,414] They are composed of transmembrane proteins and belong to a multigene family with high sequence homology among various species.[415] During implantation, a local induction of Cx26 expression, which is restricted to the luminal epithelium surrounding the implantation chamber, is observed in rats and mice.[416,417] In addition, expression of Cx26 is significantly increased after induction of decidualization. Local increases in Cx26 transcript in response to embryo recognition are ER independent during implantation and decidualization.[418] Thus, endometrial connexin expression could be under regulation by two distinct signaling pathways: Cx26 can be induced by estrogen via ERα, or it can utilize an ER-independent signaling pathway during embryo implantation and decidualization. The physiological role of these respective signaling pathways at the local level of the uterine epithelium upon embryo recognition remains to be investigated.

Matrix Remodeling and Angiogenesis

Angiogenesis, a process by which new blood vessels develop from preexisting vessels, and tissue remodeling are two hallmark events during implantation and decidualization. The changing endocrine state of the female during the reproductive cycle and pregnancy results in extensive remodeling of uterine tissues.[258,419] For example, various basement membrane components, such as type IV collagen, laminin, fibronectin, and proteoglycans, in the human uterus undergo changes throughout the menstrual cycle and pregnancy.[419] Likewise, the ECM components undergo remodeling during mouse uterine stromal cell decidualization.[258]

Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are thought to be key mediators for matrix degradation during implantation and decidualization.[419–424] There is evidence that a balance between a select set of MMPs and TIMPs is important for implantation. Mechanisms regulating the MMP and TIMP genes during the peri-implantation period are not clear, although growth factors and cytokines including the EGF and TGFβ family members and LIF have been shown to modulate MMPs and TIMPs.[419] The cathepsin family of cysteine proteases is also implicated in implantation. Cathepsin B and L are expressed in mature, invasive trophoblast cells, and the injection of synthetic inhibitors into pregnant mice during the early phase of implantation resulted in implantation failure.[425] Altered levels of certain matrix remodeling molecules are associated with unexplained infertility and recurrent miscarriages.[426]

Under normal physiological conditions, angiogenesis primarily occurs in the uterus and ovaries of the adult during the reproductive cycle and pregnancy.[427] Increased vascular permeability and angiogenesis are crucial to successful implantation, decidualization, and placentation. A number of studies provided indirect and descriptive evidence for the potential roles of estrogen

and P_4 in these processes in various species.[427–430] These studies primarily examined the changes in the whole uterus of the expression of a number of gene products known to regulate vascular permeability and angiogenesis, including vascular endothelial growth factor (VEGF) and its receptors, without investigating the angiogenic status of the uterus. Thus, in vivo roles for estrogen and P_4 in uterine angiogenesis are not fully appreciated.

VEGF, originally discovered as a vascular permeability factor,[428] is also a potent mitogen for endothelial cells and a key regulator of vasculogenesis and angiogenesis.[431] Targeted disruption of even one allele of the VEGF gene results in embryonic death in utero during midgestation with aberrant blood vessel formation.[432,433] Differential splicing of the VEGF gene generates several VEGF isoforms in both humans and mice; $VEGF_{121}$ and $VEGF_{165}$ are the predominant isoforms in humans, while $VEGF_{120}$ and $VEGF_{164}$ are the most abundant isoforms in mice.[430,434] VEGF effects are primarily mediated by two tyrosine kinase receptors: FLT1 (VEGFR1) and FLK1/KDR (VEGFR2).[435–438] FLK1 is the major transducer of VEGF signals that induce chemotaxis, actin reorganization, and proliferation of endothelial cells.[431,439,440] Another multifunctional VEGF receptor was identified as neuropilin 1 (NRP1). While NRP1 functions as a receptor for at least five different ligands, it is expressed in human endothelial cells as a $VEGF_{165}$-specific receptor.[441]

Murine VEGF isoforms and their receptors FLT1, FLK1, and NRP1 are differentially expressed in the mouse uterus in a spatiotemporal manner during implantation, and the predominant $VEGF_{164}$ isoform interacts with FLK1 and NRP1.[429,430] These results suggest that the VEGF system is important for uterine vascular permeability and angiogenesis during implantation. Others have also shown the expression of VEGF and its receptors in the uterus during pregnancy and in response to steroid hormones.[428] For example, estrogen rapidly induces uterine vascular permeability and VEGF expression transcriptionally via nuclear ER,[428] and the VEGF gene contains estrogen response elements (EREs).[442] P_4 also upregulates uterine VEGF expression via activation of nuclear PR at a slower rate.[442] Since estrogen rapidly stimulates uterine vascular permeability and VEGF expression, and since vascular permeability is considered a prerequisite for angiogenesis, it was widely believed that estrogen is a potent stimulator of uterine angiogenesis during normal reproductive processes in vivo. However, estrogen and P_4 seem to have different effects in vivo. By generating *Flk-LacZ/Esr1*$^{-/-}$ and *Flk-LacZ/Pgr*$^{-/-}$ mice to investigate the angiogenic status in *Esr1*$^{-/-}$ and *Pgr*$^{-/-}$ mice using an Flk-reporter line, it was determined that estrogen promotes uterine vascular permeability but profoundly inhibits angiogenesis, while P_4 stimulates angiogenesis with little effect on vascular permeability. These effects of estrogen and P_4 are mediated by differential spatiotemporal expression of proangiogenic factors in the uterus.[443] Cell type-specific expression of VEGF isoforms and their receptors during embryo implantation has also been reported in mink and in primates.[150,444–447]

VEGF effects are complemented and coordinated by another class of angiogenic factors, the angiopoietins.[448] VEGF acts during the early stages of vessel development,[432,433,449] whereas angiopoietin 1 (Ang1) acts later to promote angiogenic remodeling, including vessel maturation, stabilization, and leakiness.[450–452] In contrast to agonistic functions of Ang1, Ang2 behaves as an antagonist. They interact with an endothelial cell–specific tyrosine kinase receptor, Tie2.[453] There are two additional members of the angiopoietin family, Ang3 and Ang4, but definitive biological functions of Ang3 and Ang4 remain unclear. It is now shown that while VEGF and its receptor Flk1 are primarily important for uterine vascular permeability and angiogenesis prior to and during the attachment phase of the implantation process, VEGF together with the angiopoietins and their receptor Tie2 direct angiogenesis during decidualization following implantation.[454]

PGs are also involved in uterine vascular permeability and angiogenesis during implantation and decidualization. Specifically, COX2-derived PGs participate in these processes.[454] Thus, one cause for failure of implantation and decidualization in *Ptgs2*$^{-/-}$ mice is the deregulated vascular events in the absence of COX2. The attenuation of uterine angiogenesis in these mice is primarily due to defective VEGF signaling rather than the angiopoietin system. Collectively, the results provide evidence that while ovarian steroid hormones influence uterine vascular permeability and angiogenesis during the preimplantation period, COX2-derived PGs direct these events during implantation and decidualization by differentially regulating VEGF and angiopoietin signaling.[443,454]

Using reporter mice *Ptgs2*$^{-/-}$ × *Flk1LacZ* reporter mice, it was shown that COX2-derived PGs influence uterine angiogenesis in decidualization by differentially regulating VEGF and angiopoietin signaling cascades.[454] Uterine angiogenesis in *Ptgs2*$^{-/-}$ mice is compromised due to defective VEGF, but not angiopoietin, signaling, which could be rescued by introducing exogenous PG. Since PGs coordinate VEGF and angiopoietin signaling during decidualization, one contributor of compromised implantation and decidualization in *Ptgs2*$^{-/-}$ mice could be dysregulated vascular events.

Hypoxia-inducible factors (HIFs) are intimately associated with vascular events and induce VEGF expression by binding to the hypoxia response element in the VEGF promoter. HIFα isoforms function by forming heterodimers with the ARNT (HIF-β) family members. In the uterus, expression of HIFs and ARNTs does not correlate with VEGF expression during the preimplantation

period (days 1–4) in mice. In contrast, their expression follows the localization of uterine VEGF expression with increasing angiogenesis during the postimplantation period (days 5–8). This disparate pattern of uterine HIFs, ARNTs, and VEGF expression on days 1–4 of pregnancy suggests that HIFs have multiple roles in addition to the regulation of angiogenesis during the peri-implantation period. Steroid hormones also differentially regulate HIFs, with P_4 primarily upregulating uterine HIF1α expression while estrogen transiently stimulates that of HIF2α.[455] The definitive role of HIFs in regulating uterine angiogenesis warrants further investigation.

ADVERSE RIPPLE EFFECTS ARISING FROM EARLY-PREGNANCY EVENTS

Pregnancy is a feedforward program, and any disturbances to its normal course will terminate pregnancy at the time of insult or propagate defects throughout pregnancy. Compromised pregnancy outcome could result from adverse ripple effects originating from the initial insult during early pregnancy or from independent adverse effects during specific stages. Thus, determining the cause of late-stage disturbances warrants assessment of early defects. For example, dysregulation of HB-EGF, shown from studies in the mouse to be a molecular link between the embryo and luminal epithelium prior to and during attachment (as discussed in this chapter), is associated with preeclampsia, high blood pressure, and elevated urinary protein excretion in humans,[456] and presents clinical relevance that early defects can lead to pathological states later in pregnancy.

Adverse Consequences of Defects in Attachment or Implantation

Several knockout mouse models have been identified to better understand the propagation of early defects throughout the course of pregnancy. The first evidence of adverse ripple effects was shown in mice lacking *Pla2g4a* (cPLA2α) (Figure 38.8).[47] cPLA2α produces arachidonic acid from membrane phospholipids required for PG synthesis, primarily by COX2. *Pla2g4a* is co-expressed with *Cox2* in the uterus. Implantation in *Pla2g4a*$^{-/-}$ females occurs beyond the normal window of implantation (deferred implantation), leading to adverse ripple effects that are reflected in embryo crowding, retarded feto-placental growth, increased rate of resorption, and smaller litter size.[47] This phenotype is consistent with those of physiological experiments in which blastocysts were transferred into wild-type recipient uteri beyond the anticipated time of implantation.[47] cPLA2α is also expressed in the human endometrium,[457] suggesting its conserved role.

A similar phenotype was reported in mice deleted of *Lpar3* (LPA3), a receptor for lysophosphatidic acid.[48] Resemblance in phenotypes between *Pla2g4a*$^{-/-}$ and *Lpar3*$^{-/-}$ females is ultimately attributed to reduced production of COX2-derived PGs and thus the timing of implantation. Indeed, a similar observation was noted in *Ptgs2*$^{-/-}$ mice, depending on the genetic background.[369] Collectively, these findings provide evidence that the LPA3–cPLA$_2$α–COX2 signaling axis is essential for on-time implantation; an aberration of this signaling pathway defers initial attachment and perpetuates adverse effects throughout the remainder of pregnancy. Although administration of PGs rescues deferred implantation seen in *Pla2g4a*$^{-/-}$ or *Lpar3*$^{-/-}$ females, it does not rescue embryo spacing as embryo crowding still persists.[47,48]

There is evidence that haploinsufficiency of certain genes can disrupt normal physiological functions. One example is adrenomedullin (ADM), which is present in numerous tissues, including reproductive organs. ADM is secreted by decidua and trophoblast cells.[458,459] In humans, ADM levels are high in pregnancy but return to baseline levels after parturition.[460] Altered ADM levels in the feto-placental tissues are associated with spontaneous abortion, gestational diabetes, and preeclampsia.[460] In mice, *Adm*$^{-/-}$ fetuses die in utero due to placental insufficiency resulting in failure of neural tube closure, hydrops fetalis, and cardiovascular malformation.[49] Earlier studies have shown that *Adm*$^{+/-}$ mice have normal implantation but exhibit embryo overcrowding and placental anomalies, fetal growth restriction, and shallow invasion of spiral arteries indicative of preeclampsia.[49] However, it was later found that *Adm* is expressed in the uterine luminal epithelium and stroma during the peri-implantation period, and embryo transfer experiments in *Adm*$^{+/-}$ females showed compromised uterine receptivity with reduced pinopode formation (marker of receptivity) and poor implantation rate.[50] Collectively, the results suggest that defects early in pregnancy in *Adm*$^{+/-}$ females contributed to late adverse effects.

Transcription factors also regulate the window of uterine receptivity and implantation. For instance, *Msx1*$^{d/d}$ uteri show embryo crowding and a higher resorption rate, as seen in *Pla2g4a*$^{-/-}$ or *Lpar3*$^{-/-}$ females.[151] Notably, while PG signaling is present throughout pregnancy, *Msx1* is transiently expressed in the receptive uterus. This suggests that deficits even at the stage of receptivity can interfere with developmental programming in pregnancy. Uterine deletion of *Klf5* also confers deferred implantation with compromised pregnancy outcome.[213] *Klf5* is first expressed in the epithelium prior to and during blastocyst attachment; *Klf5* expression later switches to the decidua after epithelial expression becomes undetectable. These results suggest that the period spanning uterine receptivity, attachment, and decidualization is

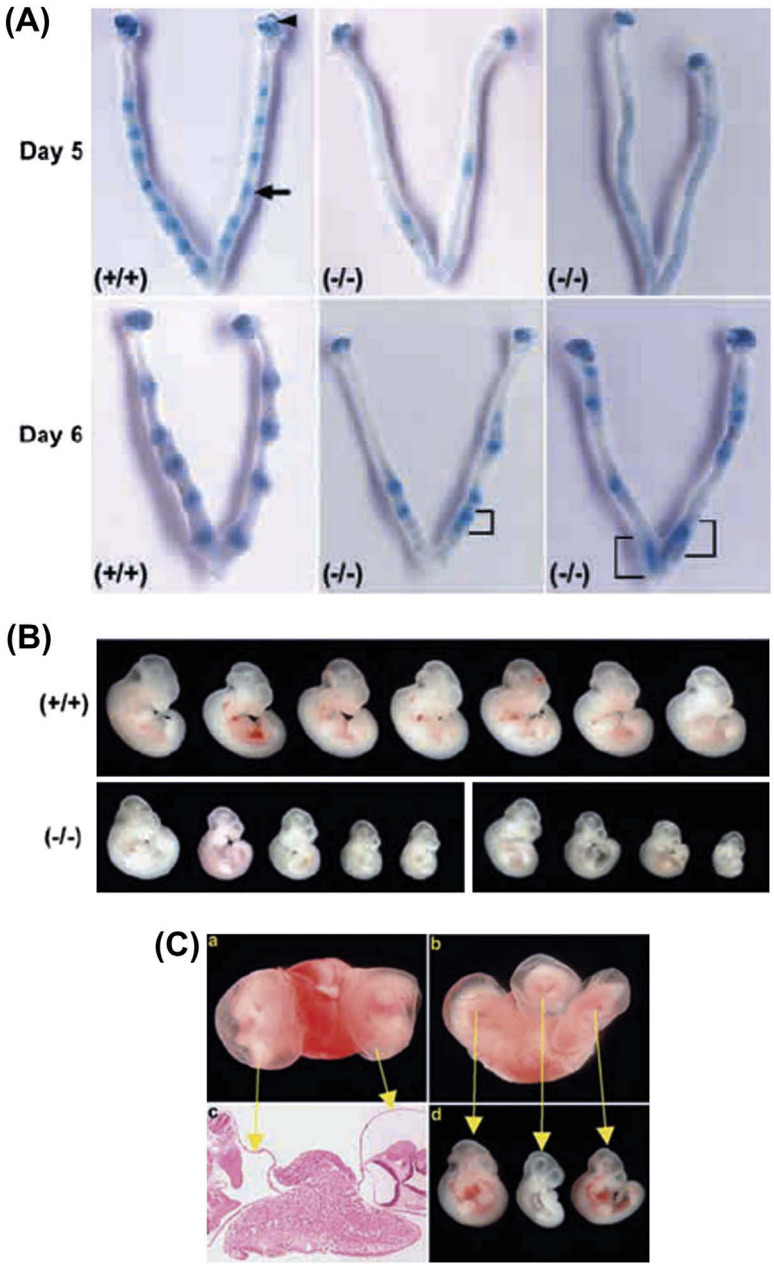

FIGURE 38.8 Defective postimplantation development in *Pla2g4a*$^{-/-}$ mice. This figure is reproduced in color in the color plate section. (A) Representative photographs of uteri with implantation sites (blue bands) on days 5 and 6. Note very few or no implantation sites on day 5, but unevenly spaced implantation sites on day 6 in *Pla2g4a*$^{-/-}$ mice. Arrowhead and arrow indicate ovary and implantation site, respectively. Brackets indicate crowding of implantation sites. (B) Photographs of embryos isolated from implantation sites of one representative wild-type and two *Pla2g4a*$^{-/-}$ mice on day 12. Note retarded and asynchronous development of embryos in *Pla2g4a*$^{-/-}$ mice. (C) Representative photographs of conjoined embryos in a placenta (a, c) and three embryos in the same decidual envelope (b, d) from *Pla2g4a*$^{-/-}$ mice on day 12. (c) A histological section of (a) with two embryos; embryos shown in (d) are from (b). Yellow arrows indicate the source of the embryos from the decidual envelope. *Source: Reprinted with permission from Ref. 47.*

susceptible to insults that later initiate adverse events during the course of pregnancy.

Adverse Effects Originating from Dysregulated Decidualization

While the section Adverse Consequences of Defects in Attachment or Implantation described poor pregnancy outcome and/or subfertility resulting from deferred implantation, defects during decidualization also lead to adverse pregnancy outcome with placentation abnormalities, intrauterine fetal growth restriction (IUGR), and difficult parturition. For example, a signature of preeclampsia is shallow spiral artery invasion by trophoblast cells.[461–463] Poor trophoblast invasion into decidua with poor remodeling of decidual arterioles is also considered a contributing factor for preeclampsia, precluding normal placentation.[51,399,464] Defective decidualization can also lead to preterm birth.[184] Mice with uterine deletion of *Trp53* ($p53^{d/d}$) show normal implantation but have increased incidence of spontaneous preterm birth with dystocia, abnormal or difficult labor, and fetal death.[184] Compromised decidualization is due to premature decidual aging resulting from more terminally differentiated, polyploid cells. These changes are signified by increased levels of pAkt, p21, COX2, and senescence-associated growth restriction. Interestingly, preterm birth in $p53^{d/d}$ females was rescued by celecoxib,

a selective COX2 inhibitor, suggesting COX2 as a critical physiological mediator of decidual senescence. Many risk factors that predispose to preterm birth, such as gene mutation, infection and inflammation, and stress, also trigger cellular senescence via mammalian target of rapamycin complex 1 (mTORC1) signaling (reviewed in Ref. 465). These results may suggest that these risk factors converge to a common pathway toward early cellular senescence of the decidua due to increased mTORC1 signaling. An mTORC1 inhibitor, rapamycin, has been shown to attenuate senescence and increase life span in mice.[466] In fact, *p53*$^{d/d}$ deciduae have high mTORC1 activity that is inhibited by rapamycin, rescuing the preterm birth phenotype.[465]

FUTURE DIRECTIONS: EVOLVING TECHNOLOGIES AND DISCOVERIES

Although considerable information regarding the roles of growth factors, cytokines, homeotic genes, transcription factors, morphogens, and lipid mediators in implantation has been generated, their hierarchical blueprint in directing uterine and embryonic function during implantation remains to be integrated. Further investigation is required to understand whether these pathways function independently, function in parallel, or converge to common signaling pathways to execute reciprocal interactions between the embryo and uterus during implantation. Thus, our understanding of the implantation process is still far from complete. Many of the genes, which are expressed in an implantation-specific manner and appear to be important for implantation, cannot be studied mechanistically because deletion of these genes results in embryonic lethality. Uterine cell type- or embryo-specific conditional deletion of genes of interest is urgently needed to better understand the definitive roles of these genes in uterine biology and implantation. Our failure to identify suitable uterine cell-specific promoters is a hindrance to achieving this objective. There is also a difficulty in identifying the critical roles of signaling molecules within a gene family because of the redundant or compensatory functions of the gene products within the family.

Strategies comparing global gene expression profiles between the implantation and interimplantation sites have identified novel genes in the implantation process. Thus, a genome-wide screening approach coupled with functional assays will help elucidate these complex signaling pathways. Experiments should be pursued to compare global gene expression patterns between wild-type and gene-deleted mouse uteri and blastocysts under defined physiological experimental conditions. The results obtained from these experiments may help uncover new signaling molecules and pathways not previously identified. The advent of microarray technology and sequencing of the mouse and human genomes have allowed us to analyze implantation-related genes in a global perspective. In mice, microarrays have been used to identify genes that show differential expression at implantation versus interimplantation sites,[467] or at implantation versus postimplantation periods,[468] or genes that are differentially regulated by changes in estrogen and P_4 signaling.[412,469–474] Comparison of the array results of implantation versus interimplantation sites to those of P_4-treated uteri versus estrogen-stimulated uteri led to identification of a number of genes with previously recognized roles in implantation and a number of new genes related to implantation and steroid hormonal regulation. Closer examination of these genes and comparing the results from similar microarray experiments and RNA sequencing may provide clues to the identity of important genes or gene families in the implantation process. In humans, microarray technology has been used to identify genes associated with the window of uterine receptivity.[277,475] A comparison of the results shows differential regulation of a small number of genes around the time of uterine receptivity for implantation. Interestingly, there are a number of genes that are also differentially expressed in mouse models of implantation and human uterine receptivity.

Gene regulation in the context of implantation can be further defined by studying noncoding RNAs, including long-noncoding RNAs, piwi-interacting RNAs, microRNAs (miRNAs), and small interfering RNAs, with exploitation of the parallel leaps in sequencing technology. Although expression analysis of these RNAs has been periodically attempted, their function in the uterus during implantation remains relatively unanswered. For example, COX2's dynamic expression pattern is refined posttranscriptionally by miRNAs, namely, mmu-miR-101a and mmu-miR-199a,[476] and there is evidence for additional uterine miRNAs in implantation.[477] Until more information is gathered regarding their regulation and mechanism of action, the function of these noncoding RNAs in implantation will remain limited. Nonetheless, this is an exciting area of research with potential to have great bearing on the dynamic changes seen during implantation.

Currently, emerging techniques in proteomics are also being applied to the understanding of uterine biology.[478,479] Proteomics is a powerful tool to map cellular protein profiles at a defined physiological state. Use of this technology has been sporadic to study implantation biology. Recent advances in in situ mass spectrometry provide opportunities to generate a spatiotemporal map of proteins and their modifications (Figure 38.9). This technology should be more widely used to generate information at specific stages of pregnancy.[480,481] With advances in high-efficiency mass spectrometry

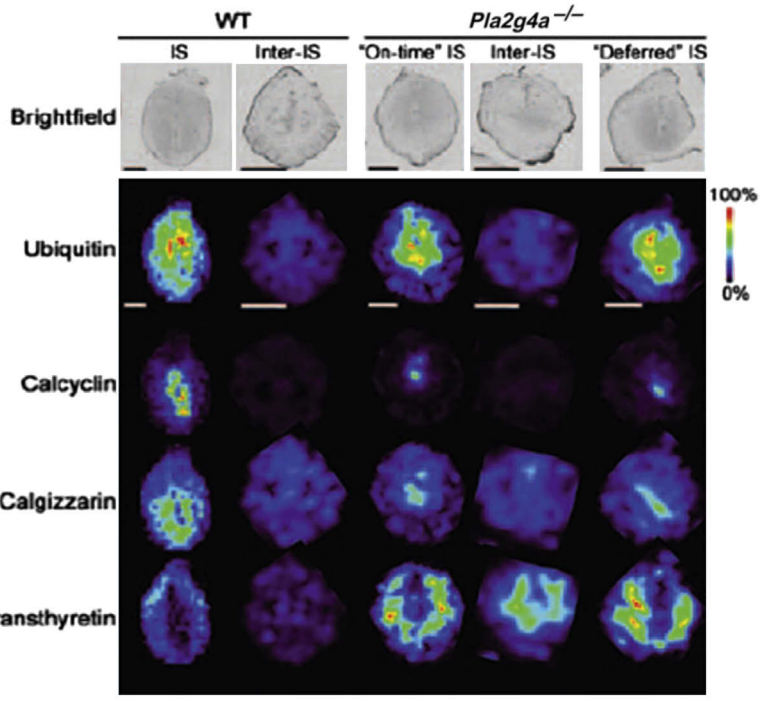

FIGURE 38.9 Proteome profiles differ between WT and *Pla2g4a*$^{-/-}$ uteri on day 6 of pregnancy, regardless of implantation timing. This figure is reproduced in color in the color plate section. Optical images of a WT implantation site (IS) and interimplantation site (inter-IS) and *Pla2g4a*$^{-/-}$ deferred and on-time IS (*upper panel*). Bar, 670 μm. Ion intensity maps are shown below their respective bright-field images. *Source: Reprinted with permission from Ref. 480.*

technology (MALDI-MS), it may now be possible to identify novel low-abundance proteins and lipid mediators involved in implantation, particularly those secreted by blastocysts. In many species, including rodents and humans, the most limiting factor is the availability of the adequate amount of tissues required for biochemical and molecular biology experiments. With the advent of microscale proteomics and genomic approaches, it is hoped that more information on embryonic signals is likely to be forthcoming. In the same vein, advanced technologies in metabolomics and lipidomics can also be used to profile metabolites and lipids to assess the physiological state of the uterus during specific stages of pregnancy.

Recent advances in imaging technology should be embraced to study implantation and pregnancy events. With the availability of high-resolution live imaging and tracking systems, the development of 3D culture systems to study implantation could provide valuable information regarding dynamic changes and interplay of molecules in situ. For example, tracking systems using fluorescent tags to monitor specific proteins at the interface of the trophectoderm and epithelium can be used to monitor their interactions.

Characterization of microbiota in different systems is a subject of intense research, and the study of the host–microbiome relationship[482] is an emerging field with implications for both health and disease knowledge. For example, studies have shown correlations of specific gut microbiome with the adaptive immune system,[483] increased risk of metabolic disease,[484] and behavior.[485] Considering the significant correlation of infection and inflammation with preterm birth,[486] efforts should be directed to characterize the influences of gut and oral microbiota on pregnancy outcome. It is possible that mapping the uterine microbiome profile may allow for predictions of adverse pregnancy outcomes, provide insight into the environmental milieu of the uterus, and may even lead to the development of uterine probiotics to optimize uterine health and fertility.

The genome–epigenome interaction and passage of nongenomic information through generations are also subjects of intense research. Environmental endocrine disrupters or composition of maternal diets during pregnancy can also have transgenerational consequences on offspring's health, ranging from lipid metabolism and sex determination, through epigenetic changes.[487–489] More mechanistic approaches are needed to understand the role of the uterine epigenome. It has been reported that transposable elements are differentially regulated in decidualization.[490]

Several limitations, including ethical restrictions in using human blastocysts, preclude the study of direct embryo–uterine interactions. In this respect, renewable sources of oocytes and spermatogonia using inducible pluripotent stem cell (iPSC) technology have recently been identified,[491,492] and several reports of renewable in vivo sources of oocytes have been generating attention and debate.[493,494] These avenues can potentially provide the basic tools to develop in vitro culture systems to study aspects of human implantation. Interestingly, the generation of trophoblast stem cells to embryonic stem cells and vice versa has been reported in mice.[495,496]

CONCLUSION

Infertility and rapid population growth are two pressing global reproductive health issues. The processes of preimplantation embryo development and uterine preparation for implantation are two major determinants for reproductive success. Basic and clinical research to better understand these events is important in order to help alleviate problems of female infertility, improve fertility regulation in women, and lead to the development of new and improved contraceptive approaches. Interactions between the major uterine and embryonic cell types with respect to endocrine, paracrine, juxtacrine, and autocrine factors during implantation are extremely complex.[153–155] Thus, exploring and defining the molecular landscape during the critical time of implantation necessitates well-thought-out experimental designs in the context of both embryonic and uterine contributions to formulate a more meaningful blueprint. As Boving wrote,[497] "The conceptus and uterus have mastered everything they need to know about implantation. We scientists have not." This mission is not easily achievable in humans due to experimental difficulties, ethical considerations, and current restrictions on research with human embryos. Therefore, model systems will continue to be used for studying embryo–uterine interactions during implantation. In addition, efforts should be directed to establish reliable in vitro systems to study implantation, which are not currently available. However, experiments using endometrial biopsy samples to identify molecules associated with human uterine receptivity (window of implantation) during the menstrual cycle with respect to changing estrogen and P_4 levels should continue to be pursued to better understand this process in humans.

Although the mechanics and cellular architecture of the implantation process vary, certain basic features are common to many species. For example, implantation occurs at the blastocyst stage, there is a defined "window" of uterine receptivity for implantation, a reciprocal interaction between the blastocyst and the uterus is essential for implantation, and a localized increase in uterine vascular permeability occurs at the site of the blastocyst during the attachment reaction. Identification and characterization of signaling pathways in these steps may elucidate a unifying scheme relevant to understanding the mechanism of human implantation.

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CHAPTER

39

Placenta and Placental Transport Function

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INTRODUCTION

The human placenta is a disc-like organ that is usually circular or slightly elliptical, measuring 22 cm in diameter and 2.5 cm in central thickness, and weighing an average of 470 g at full term (Figure 39.1). For all eutherian mammals, the placenta forms the interface between the fetus and the mother, providing all functions that are essential for fetal survival, growth, and development, including regulation of gas exchange, supply of nutrients, removal of waste products, production of essential hormones, and establishment of immunological defense.[2]

The name *placenta* means "cake" in Latin, and it is also derived from *plakous*, which means "flat cake" in Greek. It was first introduced in the sixteenth century by Realdo Columbo at the University of Padua in Italy.[3] The role of the placenta in providing nutrients to the developing fetus has been known since the fifth century BC, yet it was initially assumed to reflect direct communication between the fetal and maternal blood. The recognition of separate maternal and fetal blood circulations was attributed to William Harvey in the seventeenth century, a concept later proven by William and John Hunter in the eighteenth century, who used molten wax to demonstrate utero-placental vessels and separated circulations.[4] Weber and Dalrymple were the first to describe the placental villi and their capillary structure, followed by the identification of two trophoblast layers by Langhans in 1882. Advances in chemistry, microscopy, and blood flow physiology during the first half of the twentieth century led to the initial discoveries of the placental biosynthetic functions, and the detailed description of blood flow in the intervillous space by Ramsey and Donner.[5]

The placenta is made of cells derived from the fetus. On the side of the placenta facing the fetus, the umbilical cord is usually inserted near the center of the placental disk. The umbilical cord delivers blood from the fetus to the placenta via two umbilical arteries. Blood returns to the fetus via the umbilical vein. The remainder of the fetal side (chorionic plate) of the placenta is covered by an amnion. The maternal side of the placenta (basal plate) directly surfaces the maternal decidua, the lining of the uterus during pregnancy. This side of the placenta is characterized by grooves that divide the maternal-facing surface into 10–40 lobules (cotyledons), each connected to septa within the placenta. These lobules usually harbor one or several villous trees (Figure 39.1), but they are not autonomous, and two cotyledons are frequently supplied by the same villous tree. Although, in general, a deviation of placental shape from a circle is deemed functionally insignificant, recent data suggest an association between elliptical placentas and low birth weight.[6] Similarly, an eccentric umbilical cord insertion is associated with fetal vessel abnormalities.[7]

The main functional unit within the human placenta is the villus. The outermost layer of each villus is the multinucleated syncytiotrophoblast, which is bathed in maternal blood. Immediately subjacent to this layer are the mononucleated cytotrophoblasts, which near term form a discontinuous layer. The stroma of each villus is formed by fibroblasts and placental macrophages (Hofbauer cells). Within the stroma are the fetal blood vessels, surrounded by endothelial cells. The extravillous trophoblasts populate the placental septa and the chorionic and basal plates. Extravillous trophoblasts also invade the maternal decidua and inner one-third of the

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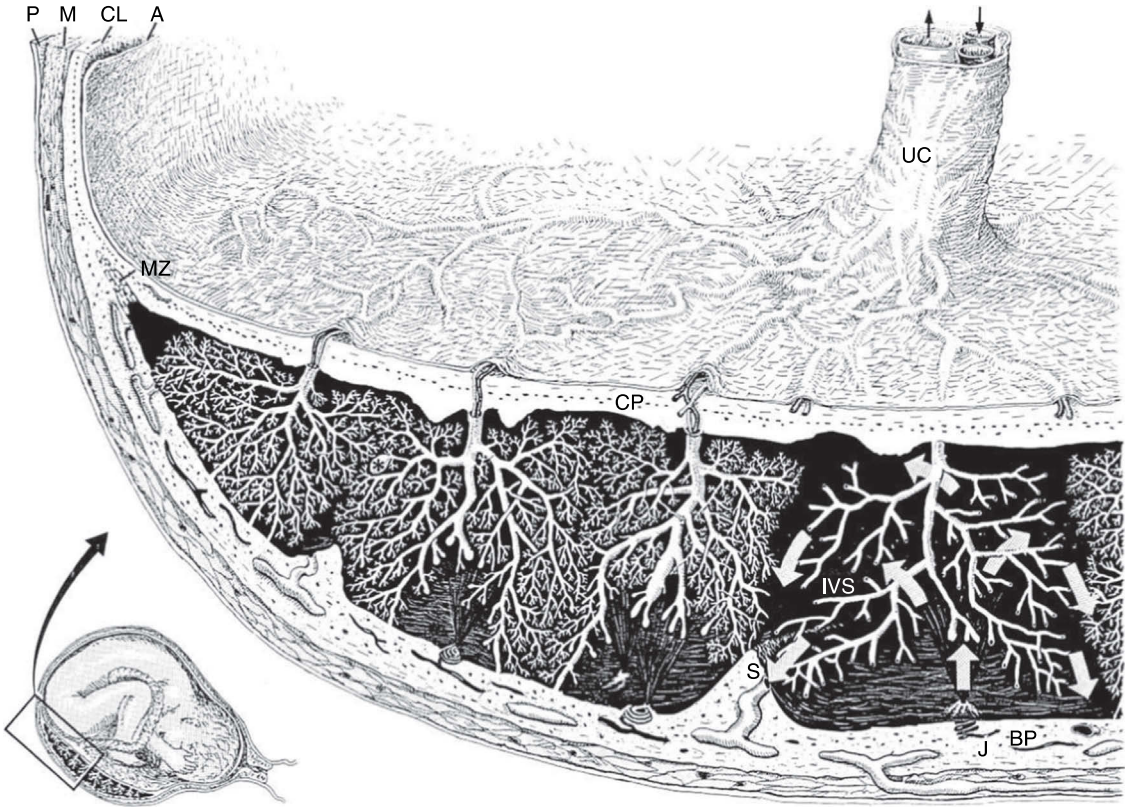

FIGURE 39.1 A diagram of a term human placenta. Shown are villous trees, arranged around maternal arterial blood inflow regions. P: perimetrium; M: myometrium; CL: chorion laeve; A: amnion; MZ: marginal zone between the placenta and fetal membranes with obliterated intervillous space; S: septum; J: junctional zone; BP: basal plate; CP: chorionic plate; IVS: intervillous space; UC: umbilical cord. *Source: Reproduced with permission from Ref. 1.*

myometrium, where they provide anchorage and support to the vasculature, and invade the blood vessels to enhance vessel dilation and ensure placental perfusion.[8]

COMPARATIVE PLACENTATION

Although all eutherian animals share the principle functions of the placenta, marked species differences exist with respect to structure and physiology, rendering the comparison of the human placenta with placentas of other animals rather complex.[9] Consequently, there is no single animal placenta that can fully capture the biology of the human placenta. Whereas the mouse pregnancy is commonly used as a model system for certain aspects of human pregnancy, this mainly reflects the ease of performing genetic manipulations in the mouse, the low cost, and the fact that the *maternal–fetal barrier interface* in the mouse placenta, like its human counterpart, is hemochorial (see the fourth criterion, discussed in the next paragraph).

Several criteria are used to define the placenta of different species. A first, central criterion is *placental vascularity*, which defines the original source of chorion vasculature. In the choriovitelline placenta, found in many marsupials, the vessels are derived from the extraembryonic mesoderm that covers the yolk sac. In most mammalian organisms, the placenta is chorioallantoic, with placental vessels originating in the allantois, the hindgut diverticulum that develops into the umbilical cord. A second criterion is *placental shape*, defined by the site of interaction of the maternal and fetal tissues. For example, in the pig's diffuse placenta, the interaction takes place over the entire chorionic sac. In the cotyledonary placenta, found in ruminants, maternal–fetal interaction is mediated within caruncles, located in nonglandular areas of the endometrium. Many carnivores have a zonary placenta, characterized by a belt of placental tissue within the chorion. Most rodents, as well as primates and humans, exhibit a discoid placenta. A third criterion is *maternal–fetal interdigitation*, which is defined by the characteristics of the maternal–fetal interaction structure. These interfaces are summarized in Figure 39.2. Notably, while the human placenta features a three-dimensional treelike villous structure, the mouse and many other rodents exhibit a labyrinthine interface, with a complex interdigitated surface for fetal–maternal interaction. A fourth criterion is the *maternal–fetal barrier interface* (Figure 39.3), which plays a pivotal role in defining the placental transport function.[9] This barrier can be invasive or noninvasive. In the noninvasive

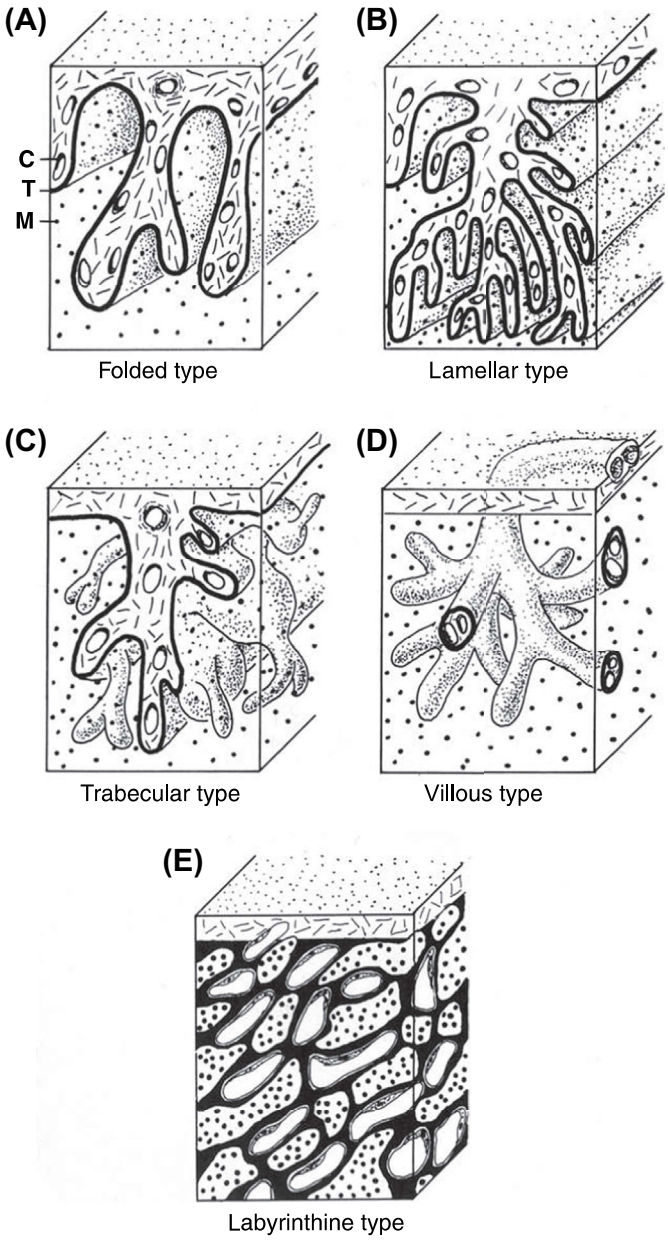

FIGURE 39.2 Examples of interdigitation between the maternal and fetal tissues at the maternal–fetal interface. The type of interdigitation may be (A) folded, (B) lamellar, (C) trabecular, (D) villous, or (E) labyrinthine. M: maternal tissue or maternal blood; T: fetal trophoblast (black); C: fetal capillaries and fetal connective tissue (stroma).

epitheliochorial interface (Figure 39.3(A)), the trophoblast is attached to the uterine epithelium but does not invade into it. In a similar noninvasive synepitheliochorial interface, the fetal binucleate cells fuse with the epithelium, yet without invasion. In the endotheliochorial interface (Figure 39.3(B)), trophoblast invasion leads to destruction of the uterine epithelium, thus reducing the barrier between the fetal and maternal vessels. The invasive hemochorial interface, characterized by direct contact between trophoblasts and the maternal vessels, is further defined by the number of cell layers separating the maternal and fetal blood, with a hemotrichorial interface (Figure 39.3(C)) that characterizes the mouse placenta, and a hemodichorial interface (Figure 39.3(D)) that characterizes the human placenta in early pregnancy.[10] In late human pregnancy, however, the placenta is partly hemomonochorial, because the cytotrophoblast cell layer is discontinuous (Figure 39.3(E)). A fifth criterion is the relative direction of the *maternal and fetal blood flow*.[9,11] When blood flows are concurrent, the maternal and fetal bloodstreams run in the same direction, whereas in the commonly found countercurrent flows, the maternal and fetal bloodstreams run in opposite directions. A mixture of the two types of flows results in cross-current flows. A more complex flow pattern is found in the human villous tree, where multivillous blood flow results in a variable flow of all types. Lastly, the *yolk sac* defines the transient need of eutherian animals for nutrients and other exchange functions prior to the formation of the functional placenta. In certain species, such as rodents, the yolk sac inverts and remains functional for the remainder of the pregnancy, where the yolk sac's endodermal lining apposes the uterine epithelial glands, enabling the absorption of large proteins, immunoglobulins, and lipid derivatives throughout pregnancy.[9,12]

HUMAN PLACENTAL MORPHOLOGY

The Placental Villus

The villus is the basic functional unit within the human placenta (Figure 39.1) and is the key site for exchange of gases, nutrients, and waste between the mother and the fetus. The trophoblast is located in the periphery of each villus, with the multinucleated syncytiotrophoblast in direct contact with maternal blood (Figure 39.4). The syncytiotrophoblast maintains polarity, with a microvillous plasma membrane (MVM) facing the maternal blood on the apical side, and a basal plasma membrane (BM) located adjacent to cytotrophoblasts and the basement membrane. It is not surprising that the syncytiotrophoblast MVM, interfacing directly with the maternal blood, harbors receptors to many types of plasma proteins, growth factors, immunoglobulins, and other soluble ligands, all directly linked to intracellular signaling cascades.[15] Subjacent to the layer of syncytiotrophoblast are the cytotrophoblasts, which usually lack direct contact with the maternal blood. The cytotrophoblasts are attached to a basement membrane that is composed primarily of type IV collagen, heparin sulfate, and fibronectin. The mononucleated cytotrophoblasts form a continuous layer in the first trimester, resulting in a complete trophoblast bilayer. Later in gestation, the cytotrophoblasts continue to proliferate yet become more

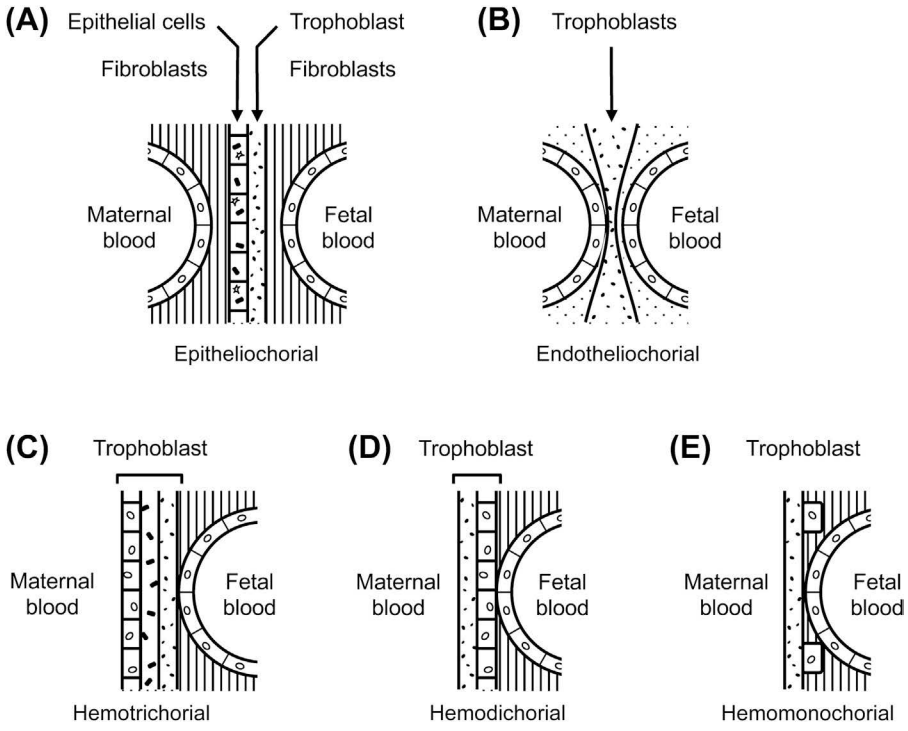

FIGURE 39.3 Examples of the maternal–fetal barrier in the placenta. Panel (A): Epitheliochorial placenta, with six layers separating the maternal and fetal blood. From left: Maternal capillary endothelium, maternal endometrial connective tissue, maternal endometrial epithelium, fetal trophoblasts, chorionic connective tissue, and fetal capillary endothelium. Panel (B): Endotheliochorial placenta, where the trophoblast is in direct contact with maternal capillary endothelium. Panel (C): Hemotrichorial, where the trophoblast is directly bathed in maternal blood. Panel (D): Hemodichorial, which is the same as (C), but with only two layers of trophoblasts. Panel (E): Hemomonochorial, which is the same as (D), but with only one layer of syncytiotrophoblast and interspersed cytotrophoblasts.

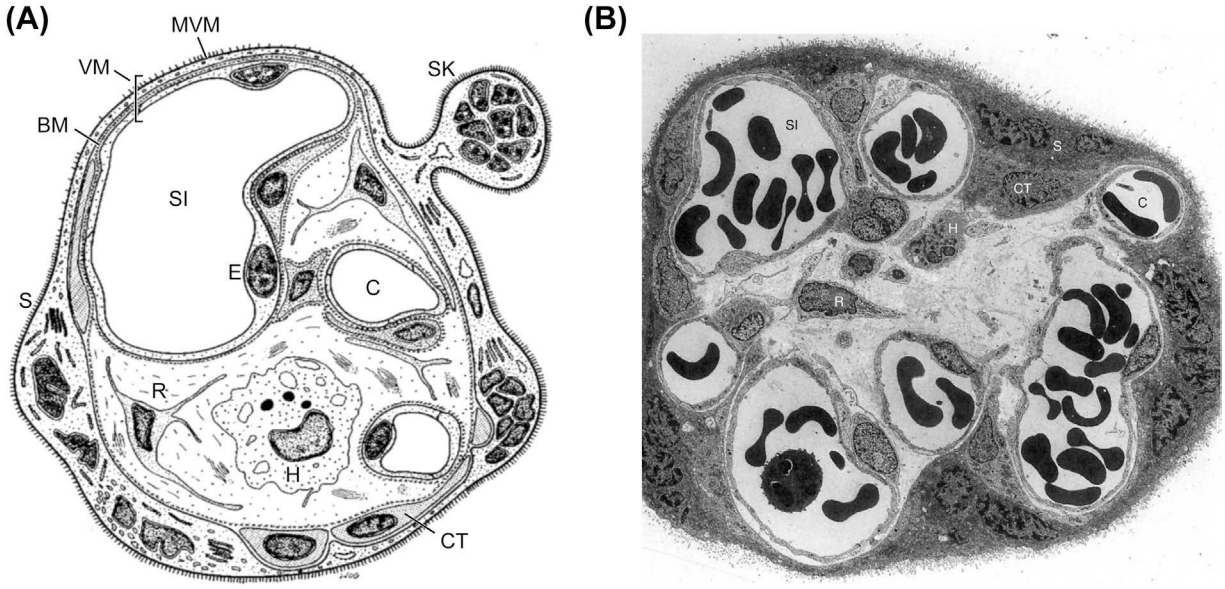

FIGURE 39.4 A terminal villus, shown in cross-section. Both panels illustrate the narrow interface between the maternal and fetal compartments. Panel (A): A schematic showing the key structural elements of the syncytiotrophoblast. *(Source: Reproduced with permission from Ref. 13.)* Panel (B): Electron micrograph of a typical, terminal villus (magnification ×2000). Abbreviations (not all shown in the two panels): VM: vasculosyncytial membrane; MVM: microvillous membrane; S: syncytiotrophoblast; CT: cytotrophoblast; C: capillary; SI: sinusoid; H: macrophages (Hofbauer cells); R: stroma with fibroblasts; E: endothelial cell; BM: basal membrane; SK: syncytial knot. *Source: Reproduced with permission from Ref. 14.*

sparse and noncontinuous, contributing to 10–15% of the total trophoblastic volume.[16] The balance among cytotrophoblast proliferation, differentiation, and apoptosis is influenced by diverse signals, such as hypoxia,[17–19] and is exquisitely orchestrated by trophoblastic proteins, growth factors, and other molecular signals, many of which have been previously interrogated in the mouse.[20–25] Differentiation of trophoblasts is characterized by fusion into syncytia, a process that occurs normally throughout pregnancy and is highly regulated by ligands such as endothelial growth factor (EGF), cyclic adenosine monophosphate (cAMP), and human

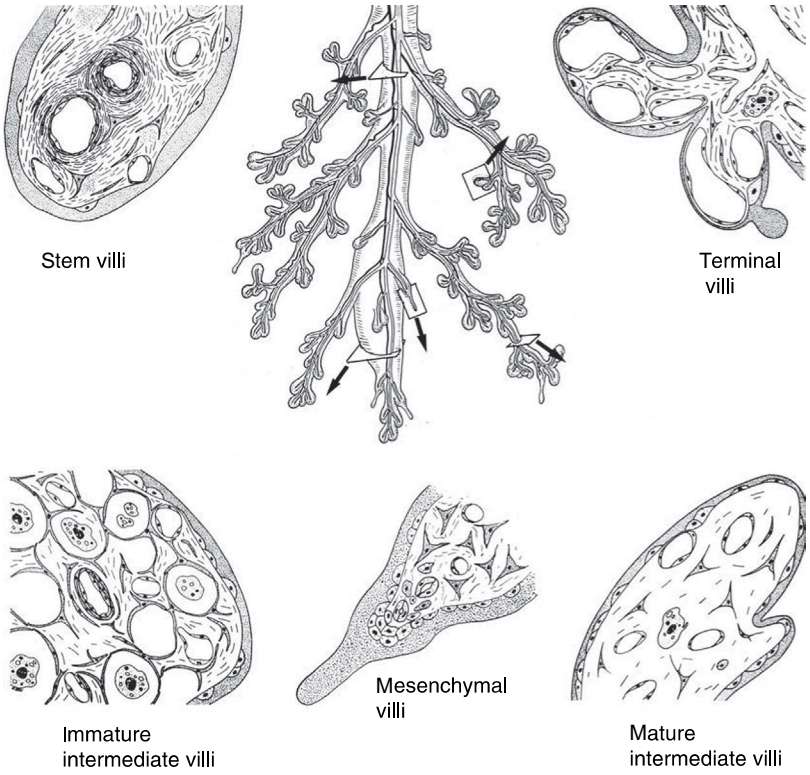

FIGURE 39.5 A simplified representation of the peripheral part of the mature placental villous tree, together with a typical cross-section depiction of different villous types. See text for details. *Source: Reproduced with permission from Ref. 1.*

chorionic gonadotropin, and by steroid hormones and genetic and epigenetic pathways.[26–30] Fusion of trophoblasts involves mechanisms that play a role in cellular apoptosis, with caspase 8 activation and the flipping of phosphatidylserine from the cell membrane inner leaflet to the outer side.[31,32] Indeed, apoptosis is commonly observed in human syncytiotrophoblasts, likely mediating the formation of clustered nuclei in syncytial knots, with subsequent shedding of cell fragments and apoptotic bodies into the maternal circulation. This process occurs more commonly in the third trimester of human pregnancy.[31,33–36] Syncytin 1, expressed from a human endogenous retrovirus, is a membrane glycoprotein that exhibits fusogenic activity in many cell types and plays a key role in mediating the fusion of mouse and human placental trophoblasts.[37,38] Syncytin is highly expressed in trophoblasts, along with its syncytin receptor, amino acid transporter 2 (ASCT2).[39,40]

The stroma of placental villi includes fibrous tissue comprising fibroblasts along with reticular, collagen, and elastic fibers.[41] At the base of the stem villi (discussed further here), these fibers blend with extravascular smooth muscle cells, creating regulatory myofibroelastic units that are believed to provide tensile support to stem villi. The stroma also includes placental macrophages (Hofbauer cells), which are fetal-derived macrophages that likely originate early in gestation from villous mesenchymal cells, and later from fetal bone marrow–derived macrophages.[8,42,43] In addition to villous immunological defense, these cells participate in the modulation of vasculogenesis and trophoblast differentiation. Placental villi also harbor fetal blood vessels, from small sinusoids in terminal villi to larger arteries and veins in stem villi. Arteries and arterioles within stem and intermediate villi are characterized by a smooth muscle coat and by an adventitia that blends with the stromal connective tissue. During the last third of pregnancy, the terminal villi capillaries are supported by a basement membrane (Figure 39.4).

The Placental Villous Tree

Early during placental development, the chorionic plate trabeculae give rise to placental lobules that form villous trunks.[44] These trunks gradually bifurcate to form either terminal villi within the intervillous space or anchoring villi at the basal plate. In general, as the placenta matures during pregnancy, some of the early villi morph into more differentiated tissues, characterized by smaller villi and narrower intervillous space. Several types of villi exist within the placenta, and are characterized by size, stromal components, and vessel structure (Figure 39.5). *Stem villi* (diameter: 80–3000 μm) are characterized by a compact fibrous stroma, with large, centrally located arterioles and venules. These villi are unlikely to participate in feto-placental exchange and largely provide tensile support to the villous tree.[3] *Mesenchymal villi* are the precursors of more differentiated immature

intermediate villi; they are characterized by unorganized stromal and vascular structures.[45] *Immature intermediate villi* form direct continuations of stem villi, and are generally found within the central regions of the lobules.[46] They are less compact than stem villi, and their cells form fiber-free intercommunicating channels in which arterioles and venues are embedded. *Mature intermediate villi* measure 80–120 μm in diameter, and they are more differentiated, with loose stroma, scant fiber cells, and capillaries that comprise at least half the villous volume. Finally, *terminal villi* measure less than 80 μm in diameter; they form the smallest and, functionally, the most important unit that mediates maternal–fetal exchange (Figures 39.4 and 39.5). Terminal villi are characterized by a variable distance between the capillaries and the syncytial microvillous membranes, representing areas of very thin membranes (vasculosyncytial membrane; Figure 39.4) as well as areas of cell bodies and stromal tissue. The capillaries typically occupy more than 50% of the terminal villous diameter. Functionally, fetal exchange is likely to occur mainly in vasculosyncytial membranes, which measure 0.5–2.0 μm wide and are devoid of cell bodies. An extension of syncytiotrophoblasts, basal membrane and endothelial cells separate the fetal and maternal blood compartments (Figure 39.4).[47,48]

The development of the villous tree is initiated at the chorionic plate's trabeculae, with the formation of syncytial trophoblastic sprouts.[45,49] Mesenchymal villi are formed with invasion of stromal tissue, followed by capillaries, into the syncytial sprouts. Rapid growth during the first trimester leads to initial sprouting of immature intermediate villi. Placental growth slows during the third trimester, at which point placental differentiation is characterized by the development of terminal villi.[45,50] Placental blood vessel development is characterized by capillary formation within the villi on day 21 post conception, with differentiation of hemangiogenic cells within mesenchymal villi.[42] This process, which takes place in a hypoxic environment, is regulated by vascular endothelial growth factor, placental growth factor (PlGF), and their receptors.[51–56] Arterioles and venules develop within the branching villi and are connected by a paravascular network of capillaries.[49,57] The arterioles and venules transform into coiled capillary loops within mature intermediate villi and terminal villi. Capillary sinusoids form from interspersed areas of vascular dilatation that reduce blood flow resistance, which is particularly germane at the vasculosyncytial membranes.

The maternal blood reaches the intervillous space through openings of the maternal spiral arteries within the basal plate (Figure 39.1), opposite the center of each lobule. Uterine veins are located more peripherally with respect to each lobule. Blood flow from each artery is initiated at the lobular center and progresses peripherally.[11] The perfusion of the intervillous space is dependent upon the pressure gradient between the maternal uterine arteries and veins, and the resistance within the intervillous space.[49] Notably, the intervillous space is irregular, and includes lakes, clefts, and fusions among neighboring villi.[34] Most functional perfusion-imaging studies, performed using the rhesus macaque, suggested that flow changes are influenced by the fetal perfusion of the villous tree. Thus, fetal flow also impacts the maternal perfusion of the intervillous space.[58]

After early development, placental location, growth, and the depth of villous invasion can be longitudinally assessed until term using ultrasound.[59] Sonographic assessment of the placental parenchyma can be used to distinguish normal from abnormal structures, including layer thickness, cystic changes, calcifications, subchorionic fluid collection, and a snowstorm appearance characteristic of trophoblastic vesicles in molar pregnancy. Advances in Doppler ultrasound, particularly color Doppler, enable noninvasive, dynamic, and quantitative real-time assessment of placental and uterine blood flow, including a detailed flow analysis in small vascular beds. Ultrasound Doppler has also become essential for clinical management of pathological pregnancies that stem from placental dysfunction.[60,61] Another noninvasive and dynamic imaging technique that has gained momentum is magnetic resonance imaging (MRI), which provides detailed information not only on placental structure and growth but also on dynamic blood flow changes within vessels and in the intervillous space.[62]

Because placental villi mediate oxygen transport to the fetus and are subject to physiological and pathological changes in oxygen levels, it is not surprising that changes in oxygenation may impact the development of the villous tree.[63] Importantly, the effect of oxygen depends on the precise stage of villous tree development, where hypoxia in early pregnancy promotes the invasion and migration of extravillous trophoblasts toward the oxygen-enriched environment near decidual blood vessels.[63–67] Conversely, hypoxia after weeks 12–14 of pregnancy has been implicated in diverse mechanisms of injury, such as direct cellular hypoxia, occurring when oxygen delivery is diminished (e.g., hypobaric hypoxia); maternal heart or lung diseases[68–70]; tissue reoxygenation, occurring during hypoxia reperfusion; and potentially even relative hyperoxia, which may occur when oxygen extraction is reduced, leading to increased delivery of oxygen further downstream. Although the precise mechanism of such injuries and placental tissue response is lacking, it is clear that each of these forms of injury, alone or in combination, may result in altered villous architecture and cellular morphology, culminating in cellular dysfunction.[71] Tissue response to these injuries also depends on the developmental stage. For example, during early villous branching, altered oxygen levels may result in reduced terminal bifurcation.

Changes in oxygenation also stimulate cytotrophoblast proliferation and/or reduced syncytial fusion, with histological changes consistent with villous ischemia and infarct.[72–74] Chronic hypoxia throughout gestation elicits an adaptive response that consists of increased vascularization of terminal villi, cytotrophoblast proliferation, villous membrane thinning, and reduced deposition of perivillous fibrin, all contributing to maintenance of maternal–fetal exchange.[68,75] Some of these responses, including increased terminal villous vascularization and thinning of the villous membrane, are also observed in chronically anemic mothers.[76,77]

The Basal Plate, Extravillous Trophoblast, and Nonvillous Support

The entire maternal–fetal interface stretches from the base of the space (Figure 39.1) at the chorionic plate to the inner third of the myometrium, termed the "junctional zone". The extravillous trophoblasts, representing trophoblasts that do not contribute to placental villi, are located within the junctional zone, and also within the chorion and chorionic plate, the placental septa in the basal plate, and the placental cell islands. Extravillous trophoblasts include the interstitial trophoblast within the junctional zone but outside the vessel wall, and the endovascular trophoblasts within the decidual and myometrial vessels. The interstitial trophoblasts are the common type of extravillous trophoblast late in gestation, and they appear as large, polygonal cells with a single intensely stained nucleus, embedded within fibrinoid. In the first trimester of pregnancy, there is a preponderance of small invasive interstitial trophoblasts, which gradually diminish until term.

The most superficial part of the junctional zone is the basal plate, which is found on the placental side after placental separation at delivery.[78] The basal plate develops from mononucleated extravillous trophoblasts which invade the decidua, with some cells forming the junctional giant cells, and others invading the decidua to generate the basal plate blood-derived fibrinoid (fibrin-type fibrinoid, or Rohr's fibrinoid). Other fibrinoid layers include the matrix-type fibrinoid, which is formed from extracellular matrix and is found deeper within a mix of trophoblasts and decidual cells. Another layer of blood derived, fibrin-type fibrinoid (Nitabuch's fibrinoid) is found on the most superficial surface of the basal plate, and represents the typical placental–decidual separation layer. The deeper part of the junctional zone, closer to the maternal uterine wall, is termed the "placental bed" and harbors a mixture of necrotic decidual cells and extravillous trophoblasts. Lastly, extensions of the basal plate into the intervillous space are termed placental septa, which are incomplete extensions of junctional zone fibrinoid, and harbor maternal components, including decidual cells and even small maternal veins.[79,80]

Trophoblastic cell columns connect large stem villi to the basal plate, a process that involves invasion of the basal plate by cytotrophoblasts early in pregnancy, and extends to the placental bed and the spiral arteries. These villi are therefore termed "anchoring villi". As pregnancy progresses, cytotrophoblast proliferation slows down, and the trophoblastic cell columns slowly degenerate until they are rarely seen in the term placenta. When columns at the tip of primary villi are not connected to the basal plate, the proliferating cytotrophoblasts are embedded in fibrinoid, forming trophoblastic cell islands.

Spiral arteries and veins that feed the placental bed are critical for maintenance of placental perfusion. The average number of spiral arteries in the term placenta is 100, and that of draining veins is 50–200. Blood flow to the placental bed is ensured by physiological remodeling of utero-placental arteries. In fact, prior to trophoblast invasion, there is already marked disorganization of spiral artery walls, even outside the implantation site, with endothelial vacuolization, disorganized vascular smooth muscle cells, and lumen dilation.[81] Physiological transformation is advanced by the invasion of extravillous trophoblasts near the arterial wall, causing deposition of fibrinoid. In the final stage of remodeling, the walls of the spiral arteries are invaded by endovascular trophoblasts that are derived from interstitial trophoblasts within trophoblastic cell columns.[82,83] These cells invade into the vessel wall media and intima, resulting in fixed-dilated vessels. The process affects the spiral arteries from the decidual end to the inner third of the myometrium, rendering the blood vessels dilated and incapable of constricting in response to vasotonic stimuli. This process seems to be more efficient in the center of the placenta, with reduced efficiency toward the placental margin.[84]

HUMAN PLACENTAL DEVELOPMENT

Developmental Milestones

Understanding key events during early and late placental development is essential in order to evaluate placental support to the developing embryo during different stages of pregnancy. While exceeding the scope of this chapter, important insights into pathologies related to placental function stem from examination of normal placental development. This section summarizes key steps in early and late placental development, delineated using morphological stages. Whereas many studies in this area were performed using human tissues obtained during early or late pregnancy, data using animal models

of pregnancy, including primates, enable physiological studies into development, function, and placental blood flow. Mouse models of pregnancy have been used not only in genetic, molecular, and metabolic studies (discussed further in this chapter) but also in the elucidation of principles underlying placental blood flow. For example, mouse sonographic studies, coupled with computerized tomographic imaging of mouse placental vascular casts, and even dynamic MRI, have helped to characterize placental vascular development and define functional or pathological changes in placental blood flow.[85,86]

The Prelacunar Stage

The extraembryonic trophoblast is the first cell lineage to differentiate during early blastocyst development and prior to implantation. Within 6–7 days after fertilization, the blastocyst attaches to the uterine endometrium (Figure 39.6(A) and (B); see also Chapter 38). At that point, the inner cell mass and blastocele are surrounded by a single layer of mononuclear trophoblasts, with cells overlying the inner cell mass defining the contact point with the endometrium. The trophoblasts that interface with the maternal endometrium fuse with the lateral borders of neighboring cells to form the first multinucleated syncytiotrophoblasts (Figure 39.6(B) and (C)). This process is completed by day 11 after implantation of the human embryo, when a complete syncytium surrounds the fully implanted blastocyst.[3]

The Lacunar Stage

A hallmark of this stage of development, lasting between days 8–12 after fertilization in human pregnancy, is the formation of fluid-filled spaces (lacunas) within the syncytiotrophoblast located at the implantation site (Figure 39.6(C)). These lacunas are the precursor of the intervillous spaces. Concomitantly, the endometrial epithelium regrows to cover the entire site, and the embryo begins to differentiate into defined layers, and is surrounded by a chorionic sac that includes trophoblasts and extraembryonic mesoderm. Formation of the lacunas subdivides the trophoblastic mantle into three major zones: the chorionic trophoblasts near the embryo, which will form the chorionic plate; the lacunar zone, which will develop into the intervillous space; and the trophoblastic shell, which interfaces with the endometrium to form the basal plate. At approximately day 12 of human pregnancy, progenitor extravillous trophoblasts from the chorion invade the three layers, and they extend deeply and laterally into the maternal endometrium (Figure 39.6(D)). These invasive cytotrophoblasts penetrate the uterine glands and cells around and within the spiral arteries, forming the endovascular trophoblast,[78,87] and transform the contractile spiral arteries into noncontractile, constantly dilated arteries,

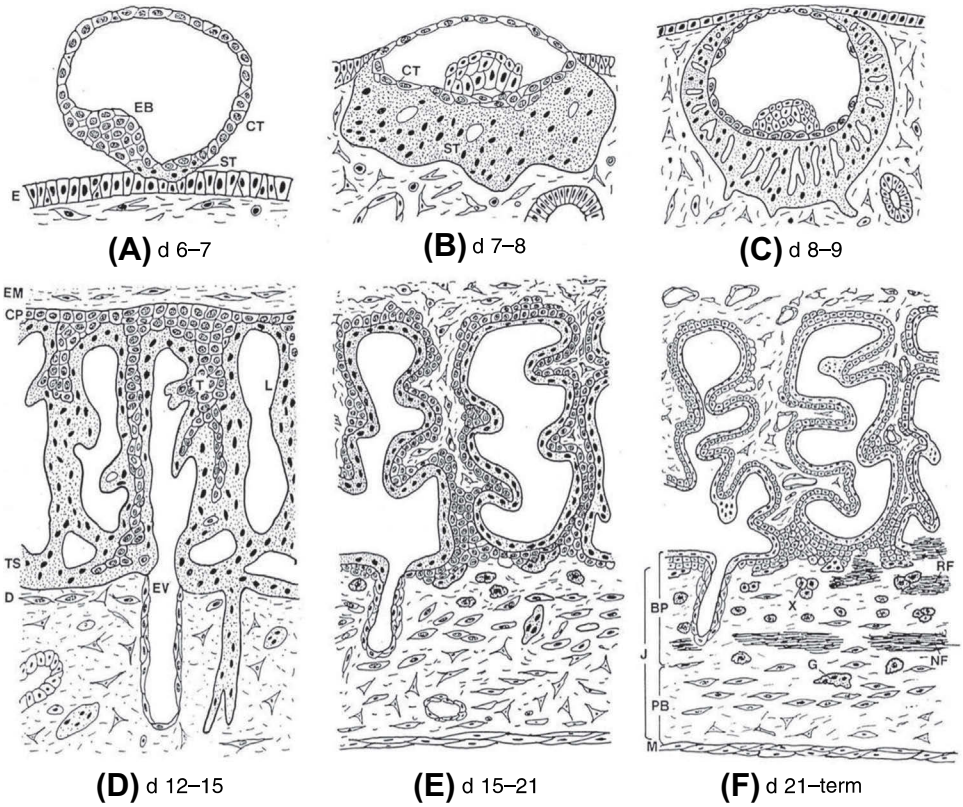

FIGURE 39.6 Simplified drawings of stages during early human placental development. Panels (A) and (B): Prelacunar stages. Panel (C): Lacunar stage. Panel (D): Transition from lacunar to primary villous stage. Panel (E): Secondary villous stage. Panel (F): Tertiary villous stage. E: endometrial epithelium; EB: embryoblast; CT: cytotrophoblast; ST: syncytiotrophoblast; EM: extraembryonic mesoderm; CP: chronic plate; T: trabeculae and primary villi; L: maternal blood lacunas; TS: trophoblastic shell; EV: endometrial vessel; D: decidua; RF: Rohr's fibrinoid; NF: Nitabuch's fibrinoid; G: trophoblastic giant cell; X: extravillous cytotrophoblast; BP: basal plate; PB: placental bed; J: junctional zone; M: myometrium.

as described in this chapter. Concomitantly, the syncytiotrophoblast interfaces with the maternal capillaries to form a direct connection between the lacunas and maternal blood, thus forming the intervillous space.[88] Once fully functional, the intervillous space fills with maternal blood and enables hemotrophic support to the embryo.

The Villous Stage

Proliferation within the cytotrophoblastic cores at the wall of the lacunas, along with trabecular elongation and branching, leads to the formation of primary villi, surrounded by the trabeculae that become the intervillous space (Figure 39.6(D–F)). The extraembryonic mesoderm penetrates into the villi to form the villous core, with cytotrophoblasts at the most distal end creating cytotrophoblastic columns, which invade toward the maternal uterine capillaries. The column cells also become a source for renewal of extraembryonic trophoblast. Proliferating hemangioblastic progenitors differentiate within the extraembryonic mesoderm to form the early first fetal capillaries,[42] which remain clogged at this point. Similarly, the trophoblastic columns invade maternal vessels, plugging their distal segments and blocking the maternal and fetal circulation, thus maintaining feto-placental hypoxia until the end of the first trimester.[63,64] The plugs start to lyse by 8–9 weeks of human pregnancy, beginning at the placental periphery and progressing centripetally (Figure 39.7). Perfusion of the placental bed results in increased oxygen tension from less than 10 mmHg to nearly 55 mmHg.[63] Doppler analysis of the intervillous space between 12 and 14 weeks of gestation confirms a fully developed villous tree manifesting a gradual decline in resistance from the umbilical vessels to the chorionic arteries and stem villi. Doppler ultrasound has also confirmed the general progression of intervillous bed perfusion from the periphery to the center, a process that is completed around 20 weeks of pregnancy.[91]

Development of Nonvillous Placental Components

The chorion lining the inner surface of the trophoblastic vesicle develops from the extraembryonic mesoderm on day 14 of human pregnancy, and it is separated at this point from the amnion by the extracoelomic cavity (Figure 39.7(B)). An expansion of the amniotic cavity brings the amnion and chorion together, until the two membranes "fuse" at 11–13 weeks post conception. This process is initiated at the cord insertion site and continues toward complete fusion within several weeks.

The connecting stalk, which is the precursor of the umbilical cord, is formed by mesenchymal cells, and it connects the amnion cavity and the extracoelomic cavity. Fetal blood vessels, which appear in the allantois after the third week of human pregnancy, fuse with the chorionic intravillous vessels to form the feto-placental circulation during the fifth week of human pregnancy. The chorionic plate of the placenta is formed by the proliferation of cytotrophoblasts and degeneration with deposition of fibrin-type fibrinoid. Fetal allantoic vessels fuse with this layer and begin to connect with vessels formed independently within villi. In extraplacental sites, the chorion apposes the maternal capsular decidua. With growth of the fetus and amniotic cavity, these layers fill the entire

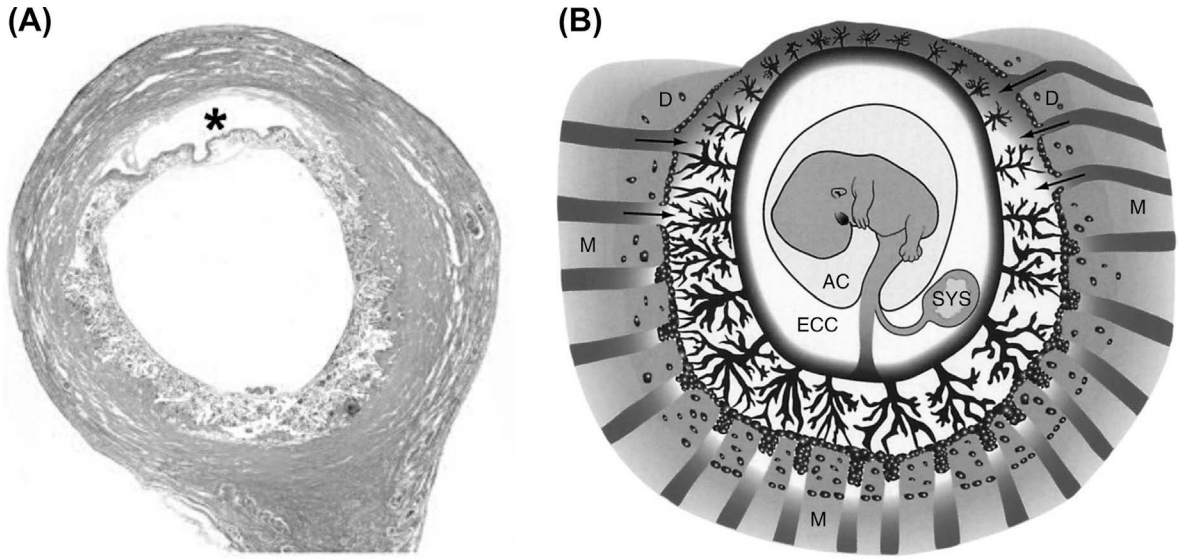

FIGURE 39.7 The perfusion of the human placenta at 8–9 weeks of gestation. Panel (A): Placenta in situ specimen showing villi over the entire surface of the chorionic sac. The villi are shorter over the anembryonic pole in association with the decidua capsularis (asterisk). *(Source: Reproduced with permission from Ref. 89.)* Panel (B): Diagrammatic representation showing the myometrium (M), decidua (D), amniotic cavity (AC), secondary yolk sac (SYS), and exocoelomic cavity (ECC). Onset of the maternal blood flow to the placenta (arrows) starts in the peripheral regions of the placenta. *Source: Reproduced with permission from Ref. 90.*

uterine cavity by approximately 15 weeks of human pregnancy, forming the attachment of the chorion with the decidua. Maternal–fetal exchange in those membranous sites communicates the fetal compartment through the amniotic cavity, not the placenta. The full thickness of the fetal membranes, which measure 0.25 mm at term, includes the innermost amniotic epithelium, with the subjacent amniotic mesoderm, the chorionic mesoderm, and the outer cytotrophoblast layer.

Molecular Control of Placental Development

Although a detailed description of the molecular processes underlying placental development is beyond the scope of this chapter, this section illustrates key regulators and principles underlying the function of these molecular regulators. Whereas the description in the preceding section focused mainly on human development, most molecular events that shape placental development have been studied in the mouse, where genetic manipulations in vivo provide definitive information on gene function that governs placental development. The mouse not only is a convenient model of genetic manipulations but also features a hemochorial, discoid (albeit labyrinthine) placenta, and many genes that regulate mouse placental development have orthologs in the human placenta. Indeed, more than 80% of genes that cause a phenotype in the mouse placenta are also expressed in the human.[92] Notably, progress in this field was largely made through reverse-genetics studies, where deletion or overexpression of a mouse gene led to an intended or coincidental placental phenotype. Because functional data about these genes in the human placenta are fairly rudimentary, the description here centers on molecular regulation of early mouse placental development. Interestingly, most regulators of mouse placental development identified thus far are not placenta specific. Thus, placental expression of many genes may represent evolutionary changes in promoter usage, the evolution of members of large family genes, or the introduction of genes into the placenta through endogenous retroviruses.[93]

Similar to human development, the first cell lineage committed within the early mouse blastocyst is the trophectoderm, which differentiates from the morula–blastocyst outer cell layer as early as embryonic day (E) 3.5, one day before implantation begins, and will contribute to placental cell lineages.[94,95] Several transcription factors are essential for early trophectoderm differentiation, including caudal-type homeobox 2 (CDX2); eomesodermin (EOMES), which is downstream from CDX2; and transcriptional enhancer factor domain family member 4 (TEAD4), which is upstream of CDX2 and coordinates the expression of CDX2.[96,97] A different set of transcription factors is expressed within the inner cell mass and maintains its cellular pluripotency.[95] The action of CDX2 and EOMES is potentiated by E74-like factor 5 (ELF5). The *Elf5* gene is methylated in the inner cell mass, preventing inner cell mass cells from entering the trophectoderm lineage.[98]

Implantation in the mouse takes place between E4.5 and E8.5 (Figure 39.8). Early proliferation of the polar trophectoderm, which is the trophoblast near the inner cell mass, is stimulated by fibroblast growth factor 4 (FGF), which also regulates CDX2 and EOMES, both essential for the development of trophoblast stem cells.[21] Unlike the human placenta, the mural trophectoderm cells within the blastocyst wall undergo a process of replication of the genome without cell division (endoreduplication) to form the trophoblast giant cells, which are critical for blastocyst attachment, adhesion, and invasion into the decidualized uterine epithelium.[99] The formation of trophoblast giant cells is regulated by several basic helix–loop–helix transcription factors, including HAND1 (heart and neural crest derivatives expressed protein 1) and STRA13 (stimulated by retinoic acid 13) that promote giant cell differentiation, and MASH2 (mammalian achaete scute-like homolog 2), which suppresses their differentiation.[100] The role and function of endoreduplication remain unclear.[101,102] Secondary giant cells differentiate from ectoplacental cone cells. The established mouse placenta has four types of trophoblast giant cells. The first type expresses proliferin (*Plf*) and is found in the wall of the spiral arteries, where it regulates artery remodeling. The second type, the parietal trophoblast giant cells, expresses prolactin family members *Plf*, *Prl3d1*, and *Prl3b1* and is found along the implantation site in direct contact with uterine decidual and immune cells, where it regulates decidualization and maternal–fetal exchange. The third type expresses *Prl3d1* and *Prl3b1*, and is found in the vascular canals, where it regulates vascular physiology. The fourth type expresses *Prl3b1* and cathepsin Q (*Ctsq*), and is located in labyrinthine sinusoids, where it regulates vessel physiology and endocrine function.[103] At late stages of development, the trophoblast giant cells secrete a wide array of hormones and paracrine signals that control pregnancy

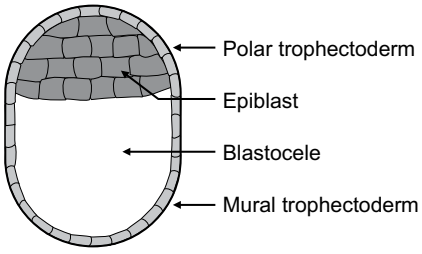

FIGURE 39.8 The blastocyst and the origin of trophoblastic cell lineage. A schematic that represents mouse embryonic day 4.5, or human embryonic day 6–7.

adaptation through regulation of vascular tone, angiogenesis, and diverse physiological functions.[103,104]

A number of genes are involved in the development and maintenance of the ectoplacental cone trophoblast and its differentiation into spongiotrophoblasts. Some of these gene products are hypoxia-responsive genes, such as the aryl hydrocarbon receptor nuclear translocator (ARNT), which partners with hypoxia-inducible factor 1 to regulate gene expression. Hypoxia or deletion of *Arnt* results in reduced spongiotrophoblast size.[100] A key player in spongiotrophoblast development is trophoblast-specific protein alpha (TPBPA, also known as, 4311). TPBPA is expressed in the early ectoplacental cone, with later expression restricted to the spongiotrophoblasts.[105] Leukemia inhibitory factor and MASH2 are critical for trophoblast giant cell differentiation into spongiotrophoblasts, and HAND1 regulates differentiation of giant cells derived from the mural trophectoderm and the ectoplacental cone.[101,106] While the precise role of spongiotrophoblasts remains to be established, this layer, which lies between the parietal trophoblast giant cells and the labyrinth, harbors specific secretory cells, such as glycogen trophoblast cells. Differentiation of more specialized cells within the spongiotrophoblast is largely unknown. Insulin-like growth factor 2 (IGF2) and the cyclin-dependent kinase 2 (P57Kip2) promote differentiation of spongiotrophoblasts into glycogen cells.[100,107]

By E8.5, the allantois comes into contact with the chorion beneath the ectoplacental cone, forming the initial blood vessel precursors. This event marks the beginning of labyrinth formation.[108] The labyrinthine zone is analogous to the human villous tree and largely mediates the maternal–fetal exchange.[107] Notably, the labyrinthine trophoblast has three layers of trophoblasts, with two inner layers of syncytiotrophoblast and an outer interrupted layer of cytotrophoblast. Glial cells missing homolog 1 (GCM1) is essential for labyrinth formation and syncytialization, with deletion resulting in midgestation death due to failure of labyrinth development.[21] Many other genes are involved in murine labyrinth formation and maintenance, such as extraembryonic, spermatogenesis, homeobox 1 homolog **(***Esx1*); peroxisome proliferator-activated receptor gamma (*Pparg*); retinoid X receptors (*Rxrs*); the Jun B proto-oncogene (*JunB*); retinoblastoma (*Rb*); and the enzymes argonaute2 (*Ago2*) and *p38*.[100,107]

Placental hormones are critical for the regulation of placental development and function. For example, chorionic gonadotropins control progesterone production, endometrial receptivity, and other essential functions.[93] Members of the prolactin family stimulate growth and lactogenic activity, and they promote placental blood flow and other aspects of adaptation to pregnancy.[104] The interferon-τ (*Ifnt*) gene regulates the production of progesterone from the corpus luteum by suppressing luteolytic signals.[109]

Diverse epigenetic, imprinting, and micro-RNA (miRNA) pathways are also critical for placental development. Genomic imprinting is a process by which one allele is epigenetically silenced in a parent-of-origin manner, and it commonly occurs through methylation, histone modification, or an RNAi mechanism. There are more than 100 imprinted genes in the human or mouse genome. Many of them are expressed in the placenta and regulate placental development and function.[110,111] In general, the parental conflict theory postulates that paternally expressed genes are designed to stimulate fetal growth, whereas maternally expressed genes tend to limit nutrient supply to the embryo in order to preserve maternal reproductive sources.[111,112] As the placenta governs transport functions that are essential for fetal growth, it is not surprising that general changes in imprinting patterns impact fetal growth.[113–115] Altered expression of selected imprinted genes that regulate placental thickness, surface area, or vascularity (e.g., *Igf2*; the maternally imprinted H19 long intergenic noncoding RNA (lincRNA); growth factor receptor-bound 10 (*Grb10*); and pleckstrin homology-like domain, family A, member 2 (*Phlda2*)) affect passive diffusion or transporter-mediated properties of the placenta, impacting fetal–placental weight (termed "placental efficiency").[116,117] Nonetheless, the correlations of placental morphology with fetal size are inconsistent, with enlarged placentas that are not associated with larger embryos in *H19-*, *Phlda2-*, and placenta-specific 1 (*Plac1*)-null mice, and smaller placentas in *Igf2P0*-null mice associated with efficient placental function, characterized by the upregulation of glucose transporters.[117] In addition, temporal or spatial expression patterns of imprinted genes may vary between biallelic expression to monoallelic expression during pregnancy.[117] Lastly, imprinted genes are commonly responsive to changes in the environment, with influences such as culture medium during in vitro fertilization, and protein and alcohol consumption, markedly affecting placental gene expression.

Similar to many other organs and tissue types, the placenta expresses diverse types of small noncoding RNAs. Among these species, miRNAs are the most abundant and represent the best characterized class.[118,119] Generally, miRNAs function coordinately in fine-tuning the expression of mRNAs and proteins that shape development and physiology. Further supporting the role of miRNAs in placental development, a mutation in mouse *Ago2*, which disables the miRNA processing machinery, causes reduced labyrinth formation and fetal growth restriction, resulting in embryonic lethality.[120] Other miRNA biogenesis proteins are expressed in human placental trophoblasts and may be germane for placenta development, yet mice deficient in these proteins exhibit

developmental defects that precede placental formation, impeding research into their role in the placenta. Although the function of most placental miRNA remains to be determined, certain patterns have emerged, including the expression of three miRNA clusters—the chromosome 19 miRNA cluster (C19MC), the chromosome 14 miRNA cluster (C14MC), and miR-371-3—which dominate the placental miRNA landscape.[121] Processes that occur in the placenta during the first trimester, such as trophoblast proliferation, migration, and invasion, are shaped by miRNA expression.[122] Specific examples include miR-378a-5p, which targets Nodal, an inhibitor of trophoblast proliferation, migration, and invasion[123]; and miR-148 and miR-152, which regulate human leukocyte antigen G expression.[124] H19, which is located in chromosome 7 in the mouse and in 11p15.5 in the human, regulates fetal and placental growth. This locus also expresses miR-675, which is expressed from the mouse placenta in late gestation (after E15.5) and attenuates cell proliferation. A deletion of miR-675 in mice increases placental size, likely acting through silencing growth-promoting IGF1 receptor (*Igf1r*).[125] Interestingly, miR-675 excision from H19 is regulated by the RNA-binding protein, human antigen R, which, in turn, is regulated by the placenta-specific miR-519.

The most abundant family of all human placental miRNAs is C19MC.[118,126] This primate-specific cluster is located on chromosomal region 19q13.41 and encodes 46 highly related miRNAs within a ~100 kb region of genomic DNA.[127] The expression of the C19MC cluster is imprinted, with expression from the paternally inherited chromosome that is regulated by a CpG-rich region located ~17 kb upstream of C19MC.[128] Although the function of the C19MC in the placenta remains to be determined, the unique abundance and regulation of placental C19MC miRNAs suggest an important role in trophoblast biology.

Unlike the insignificant role of the human yolk sac after week 10, the mouse yolk sac enlarges rapidly and forms the inverted yolk sac placenta. Early yolk sac development is influenced by the ligands FGF and retinoic acids, and the transcription factors GATA binding protein 6 and sex-determining region Y (Sry)-box 17.[129] After segregation to the primitive endoderm and epiblast, the primitive endoderm gives rise to the parietal endoderm and visceral endoderm components of the yolk sac. The parietal endoderm governs transport functions between the uterine wall and the yolk sac, and is in direct contact with the Reichert membrane, which separates the parietal endoderm from the trophoblast giant cells.[130] The visceral endoderm contributes to the intraplacental and visceral yolk sac, which is formed at E7.5 and is in direct contact with the extraembryonic mesoderm to facilitate the transport of gases, nutrients, and waste between the mother and the fetus. The mouse yolk sac remains important for protein and sterol metabolism until term.[131]

METHODS TO STUDY PLACENTAL TRANSPORT FUNCTIONS

Considering the ethical limitations on experiments in pregnant women, most mechanistic studies are based on in vitro approaches such as cultured primary human trophoblasts from the three trimesters of pregnancy, placental cell lines, and human placental explants. While clearly important, these approaches do not capture the complexity of perfusion and transport functions. Moreover, unlike most metabolically active tissues that involve the processing and partitioning of nutrients and metabolic products between tissues and blood, the placenta harbors two distinct yet intertwined circulatory systems, fetal and maternal. Placental transport and metabolic function are at the interface of two systems. Complexity is increased by the ongoing anatomical and physiological changes that occur in each of the two compartments during pregnancy, and the existence of potential competition for limited resources between the mother and the fetus. Thus, optimal pregnancy outcome depends on a homeostatic balance among metabolically active tissues within the mother and, separately, within the fetus. It is therefore clear that in vivo as well as ex vivo systems that capture the placental dual-perfusion system are critical for adequate examination of placental transport function. Such systems span diverse animal models, including genetically modified mice, ex vivo perfused placental cotyledons from humans, and ex vivo perfused mouse placentas. While the characteristics of the maternal–fetal interface are most helpful in order to understand the function of the placental interface, it should not be assumed that the mere structure, the number of layers, the blood flow, or the animal's location on the evolutionary tree, as discussed in this chapter, implies transport efficiency. It also seems simplistic to assume that placental "efficiency" can be measured in terms of a feto-placental weight ratio, which is nearly 7:1 in the human but more than 10:1 in the mouse. The following sections in this chapter summarize key observations based on single or combined approaches to analysis of placental transport function.

BASIC CONCEPTS IN MATERNAL–FETAL EXCHANGE

Pathways of Placental Transfer: Transtrophoblastic Channels

Substances can be transferred across epithelial barriers either between cells via intercellular water-filled spaces

(paracellular transport) or through cells (transcellular transport). In classical epithelia, such as the kidney and intestine, paracellular ion and solute transport is largely controlled by dynamic regulation of the permeability of tight junctions, which are multiprotein complexes connecting the apical ends of adjacent columnar epithelial cells. However, because the syncytiotrophoblast is a true syncytium, no intercellular spaces exist in the transporting epithelium of the human placenta. Thus, it is suggested that the term "transtrophoblastic channels", rather than "paracellular channels", be used for water-filled channels traversing the syncytiotrophoblast. The existence of transtrophoblastic channels is supported by physiological data obtained in several species with hemochorial placentas, demonstrating that the transplacental flux of small inert hydrophilic molecules is proportional to their water diffusion coefficients (reviewed in Ref. 132). This relationship can be accounted for by the presence of water-filled channels that are continuous with the fluid on both sides of the syncytium. Furthermore, evidence of transfer of large molecules such as alpha-fetoprotein[133] and horseradish peroxidase[134] across placental cotyledons perfused in vitro, and the recovery of fetal lymphocytes and other fetal blood cells (reviewed in Ref. 135) in the maternal circulation in vivo, are consistent with the existence of transtrophoblastic channels. On the other hand, the ability of the syncytiotrophoblast to generate and maintain significant concentration differences for a large number of molecules across the placental barrier, including Ca^{2+} and amino acids, strongly suggests that the total area occupied by water-filled channels traversing the syncytium is very small. Therefore, the primary route of transport across the placental barrier for nutrients and important ions is transcellular, in most cases mediated by specific transport mechanisms.

Importantly, for molecules that are actively transported to the fetus, the presence of transtrophoblastic channels tends to decrease net transfer by allowing a "back flux" of molecules to the maternal circulation. Furthermore, the direction of the hydrostatic pressure gradient in the human placenta in vivo is in the fetal-to-maternal direction,[136,137] resulting in a hydrodynamic water flow toward the mother. Thus, net flux from the mother to the fetus through water-filled channels requires a significant concentration difference between maternal and fetal blood as a driving force for diffusion to overcome the back flux toward the mother. The structural correlate to the transtrophoblastic channels remains elusive, yet it is possible that temporary breaks in the syncytial barrier constitute the water-filled pathway supported by the functional data. Such breaks are likely to occur in the human placenta, given the extreme thinness of the barrier in terminal villi and the mechanical forces it is subjected to by maternal blood entering the intervillous space from the spiral arteries. Indeed, trophoblastic denudations associated with fibrin deposits may be the result of such temporary breaks in the barrier.[133]

Pathways of Placental Transfer: Transcellular Transport

Maternal–fetal exchange via the transcellular route involves transfer across the two polarized plasma membranes of the syncytiotrophoblast. The transfer of a molecule across the placental barrier can be limited by the rate of blood flow on the two sides of the barrier ("flow-limited" transport) and/or by diffusion across the barrier itself ("diffusion-limited" transport). For example, the transfer of oxygen across the placental barrier is blood flow-limited because it is a small molecule with high lipid solubility, allowing rapid diffusion across the syncytiotrophoblast and fetal capillary endothelium. As a result, even short-lasting reductions in placental perfusion may affect fetal oxygenation. In contrast, the transfer of nutrients such as glucose and amino acids is predominantly diffusion-limited by the transport properties of the barrier itself. For molecules with flow-limited transport, there are generally no specific transporters in the barrier, and the transport is characterized as "nonmediated". On the other hand, for most molecules with diffusion-limited transport, there are specific transporters, binding proteins, or receptors that are expressed in the syncytiotrophoblast plasma membranes or cytosol, which increase the rate of transfer ("mediated transport").

Nonmediated Transport

The rate of transplacental transport of molecules that lack specific transport mechanisms is dependent on the physical and chemical properties of the molecules, in particular their charge, size, lipid solubility, and degree of protein binding. Thus, electrical charge, low lipid solubility, a high degree of protein binding, and high molecular weight are all molecular properties that impede passive diffusion between the two blood circulations. The same physical–chemical properties govern the nonmediated transfer of pharmaceutical drugs across the placental barrier. For example, oral anticoagulants such as dicumarol have relatively low molecular weights and cross the placental barrier quite readily, whereas heparin, which is a charged molecule with a high molecular weight, is hardly transferred across the barrier. Nonetheless, many drugs are subjected to mediated uptake, metabolism by cytochrome 450 enzymes, and/or extrusion from the syncytiotrophoblast by efflux pumps such as P-glycoprotein, limiting the value of physical–chemical properties in predicting placental drug transfer.

Mediated Transport

The transplacental transfer of most nutrients and ions is mediated by specific transport mechanisms. When mediated transport does not require energy expenditure, the transport is termed "facilitated transport" or "facilitated diffusion". The term "active transport" implies that energy is consumed, either directly (primary active transport) or indirectly (secondary or tertiary active transport). The most common pathway for mediated transport in the placental barrier is the presence of transporter proteins in the syncytiotrophoblast plasma membranes. Maternal–fetal glucose transfer is an example of facilitated transport, mediated by facilitative glucose transporters expressed in the MVM and BM of the syncytiotrophoblast. Calcium efflux across the BM, mediated by Ca^{2+}-ATPase, is an example of primary active transport. Na^{+}-dependent transport systems for amino acids, such as System A, represent a secondary active transport mechanism. Transplacental transport may also be facilitated by the expression of receptors in the MVM (e.g., the transferrin receptor, which binds the transferrin–iron complex in maternal blood, thereby facilitating the placental uptake of iron) or binding proteins in the syncytiotrophoblast cytosol (such as fatty acid–binding proteins).

Endocytosis–Transcytosis

This process constitutes a special form of transport, which involves intracellular vesicle formation by invagination of the plasma membrane on one side of the syncytiotrophoblast. Subsequently, vesicles may be transported to the opposite side of the cell, where the vesicle content can be released into the extracellular space following fusion of the vesicles with the plasma membrane. Endocytosis and transcytosis can be nonmediated in that vesicles are formed at the plasma membrane incorporating fluid and any dissolved solute (fluid-phase endocytosis). Vesicles can be transferred across the syncytium via Brownian movement. Mediated endocytosis involves the binding of a specific ligand to a receptor in the plasma membrane, which initiates the invagination of the plasma membrane. In addition, for mediated endocytosis, vesicle transfer across the cytoplasm may be facilitated and guided by specific cytoskeletal components. Endocytosis–transcytosis plays an important role in the transplacental transfer of immunoglobulin G (IgG), iron, and lipoproteins.[138]

Placental Micro- and Nanovesicles

Located at the surface of the placenta and bathed in maternal blood, the trophoblast that overlays the basement membrane and fetal capillary endothelial cells is positioned to mediate the crucial cross-talk between the maternal host and the semiallograft fetus. In addition to the release of hormones, growth factors, and other trophoblastic peptides, the microvillous membrane of the syncytiotrophoblast produces micro- and nanoparticles of diverse sizes, which are released into the maternal circulation. This shedding process may be enhanced in conditions associated with disruption of the microvillous membrane surface, such as preeclampsia (Figure 39.9).[139] The released materials include large trophoblastic fragments, single cells and syncytial knots (size generally >1 μm), apoptotic bodies of diverse sizes, microvesicles (0.15–1 μm), exosomes (40–150 nm), and nonpackaged molecules.[140] While the precise content of each of the vesicles remains to be determined, many of these vesicles seem to participate in immune signals.[141,142] Notably, exosomes are known to harbor a significant fraction of plasma miRNAs, which recently emerged as mediators of intercellular communication.[143,144] Selected placenta-specific miRNA species are found in the maternal circulation during pregnancy, and their levels rapidly decline in the first 24 h postpartum.[126,145] Pregnancy-related changes in circulating

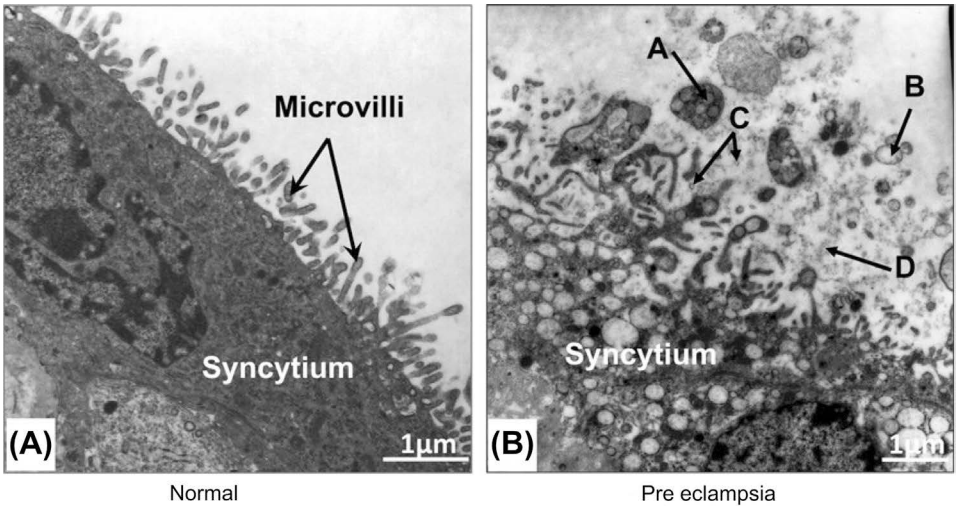

FIGURE 39.9 Ultrastructure of normal and preeclamptic syncytial microvillous plasma membrane (MVM) surface. Panel (A): Normal term syncytium showing a regular MVM. Panel (B): Syncytium from a pregnancy complicated by severe preeclampsia, showing loss of surface integrity, distorted microvilli, and shedding of debris ranging from (A) large cellular fragments containing swollen endoplasmic reticulum characteristic of apoptotic bodies, to (B) microvesicles, (C) exosomes, and (D) fine cellular material. *Reproduced with permission from Ref. 139.*

placental miRNAs are not uniform, with miR-526a increasing by 700-fold between nonpregnant women and those in the third trimester of pregnancy, but only by 2.8-fold for miR-518e.[145,146] During the first trimester of pregnancy, trophoblast exosomes may contribute to the establishment of maternal immune tolerance, possibly via impaired T-cell signaling, downregulation of natural killer (NK) cell receptor NKG2D, and enhanced apoptotic pathways through Fas ligand, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, and programmed cell death protein.[118,141,147] Whereas the transport of free miRNAs or exosome-packaged miRNAs between trophoblasts and the maternal blood is direct, transport of miRNA between the fetus and the placenta, or from the fetus through the placenta into the maternal circulation, is more intricate and involves passage through fetal capillary endothelial cells, the basement membrane, and trophoblasts.

Factors Influencing Placental Transfer: An Overview

In general, the rate of transport across the placental barrier is determined by the driving forces and the characteristics of the barrier. The extent to which these factors influence net transfer is highly dependent on each specific molecule. Differences in concentrations (ΔC) and electrical potential ($\Delta\Psi$) constitute the driving forces for nonmediated and facilitated diffusion, even though potential differences affect only molecules with an electrical charge. Water transfer via transtrophoblastic channels and the transcellular route is driven by osmotic ($\Delta\pi$) and hydrostatic pressure differences (ΔP). Water moving through transtrophoblastic channels (bulk flow) also carries solutes (often referred to as "convection"). Finally, the driving force for active transport is ATP hydrolysis.

In many species, there is an electrical potential difference between the maternal and fetal extracellular fluids (reviewed in Refs 132,148). The source of this potential difference is not entirely clear, yet evidence suggests that two oppositely oriented electrical currents, one across the placenta and the other across the decidua, generate the net maternal–fetal potential difference (PD_{mf}).[132] The transplacental potential difference is a result of active ion transport, which is the predominant transport mechanism for all main ions. The recorded PD_{mf} is significant in some species, such as the goat (−80 mV, sign indicating the polarity of the fetus with respect to the mother), the sheep (−40 mV), and the rat (+15 mV). In contrast, the PD_{mf} in the human is small (−2.7 mV) in midgestation and close to zero at term. Because the PD_{mf} is generated by the sum of the placental and decidual currents, this measurement may not provide accurate information with respect to the local potential differences at the placental barrier. In addition, potential differences across individual components of the placental barrier affect the flux of charged molecules. For example, the potential difference across the syncytiotrophoblast microvillous plasma membrane in vitro was recorded to be −32 mV early in the first trimester and −21 mV at term.[149]

The barrier properties that may influence the transfer rate of molecules are illustrated in Figure 39.10. In the term human placenta, there are only two complete cell layers separating the fetal and maternal circulations: the syncytiotrophoblast and the fetal capillary endothelial cell. Fetal capillaries in the placenta are of the continuous type, allowing the unrestricted passage of molecules of the size of glucose and amino acids between cells through intercellular spaces but partly restricting the transfer of large molecules such as immunoglobulins.[150] As described in this chapter, the syncytiotrophoblast is the main transporting epithelium of the human placenta. While in early pregnancy the cytotrophoblasts form a continuous cell layer between the syncytium and the fetal capillaries, in late pregnancy the cytotrophoblasts are relatively sparse (Figure 39.10), establishing a central role of the syncytiotrophoblast MVM and BM in determining transport.

The overall rate of transfer of a molecule is proportional to the total effective exchange surface area (Figure 39.10, #1). Thus, the villous surface area is an important determinant of placental transport capacity. Additionally, changes in blood flow influence the available effective exchange surface area, because inadequate blood flow on both sides of the barrier will lead to ineffective exchange. The passive properties of the MVM (Figure 39.10, #2) and BM

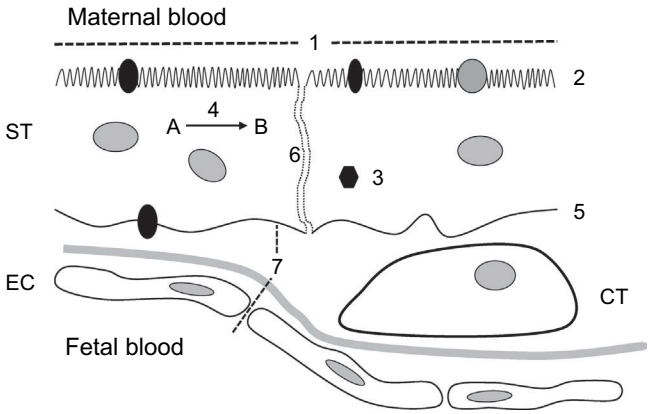

FIGURE 39.10 Properties of the placental barrier that may influence the overall transfer rate. See text for details. (1) Total effective surface area; (2) the properties of the syncytiotrophoblast microvillous plasma membrane (MVM), including passive characteristics such as lipid composition and fluidity, and expression of transporters, channels, enzymes, and receptors; (3) cytosolic binding proteins; (4) metabolic interconversion or catabolism in the cytoplasm; (5) properties of the syncytiotrophoblast basal plasma membrane (BM), including passive characteristics such as lipid composition and fluidity, and expression of transporters, channels, enzymes, and receptors; (6) transtrophoblastic channels; and (7) diffusion across the basement membrane, villous core, and capillary endothelium. ST: syncytiotrophoblast; CT: cytotrophoblast; EC: endothelial cell.

(Figure 39.10, #5), such as lipid composition and fluidity, are important determinants for nonmediated transport. The thickness of the villous membrane (i.e., the distance between the maternal and fetal blood) also influences placental diffusing capacity.[151] During gestation, the thickness of the villous membrane gradually declines principally due to expansion of the fetal capillary bed, a process that is associated with increased diffusing capacity.[152,153] The expression and activity of transporters (e.g., glucose transporters), channels (e.g., calcium channels in the MVM), enzymes (e.g., MVM lipoprotein lipase), and receptors (e.g., transferrin receptor in MVM, mediating iron uptake) in the MVM and/or BM are critical for mediated transport. Indeed, for most substances, the movement across the MVM and/or BM is the rate-limiting step in transplacental transfer. These transmembrane transport steps are also highly regulated. For some molecules, including fatty acids and Ca^{2+}, the presence of cytosolic binding proteins (Figure 39.10, #3) is essential for facilitated transfer across the syncytiotrophoblast. An additional property of the placental barrier that may influence the overall transfer rate of some molecules (e.g., glucose, lactate, and amino acids) is metabolic interconversion or catabolism within the syncytiotrophoblast (Figure 39.10, #4). Transtrophoblastic channels (Figure 39.10, #6) may mediate maternal–fetal exchange of water and solutes. Finally, molecules delivered to the fetal side of the syncytium must move across the basement membrane and capillary endothelium (Figure 39.10, #7).

PLACENTAL TRANSPORT OF SPECIFIC MOLECULES

Oxygen

Oxygen is critical not only as a substrate in fetal–placental oxidative metabolism but also for placental growth, invasiveness, and regulation of trophoblast gene expression. In response to long-term experimental reduction of umbilical blood flow or maternal hypobaric hypoxia, the fetal sheep becomes hypoxemic and growth restricted, suggesting that limited oxygen supply restricts fetal growth.[154] This hypothesis is difficult to prove because interventions to produce fetal hypoxemia likely alter a multitude of other factors, including placental blood flow, nutrient delivery, and carbon dioxide removal. However, studies in chick embryos, a model in which the effects of changes in oxygenation on the fetus can be investigated in isolation, have confirmed a role for oxygen supply in the regulation of fetal growth.[155]

Placental oxygen transfer is limited by blood flow rather than by diffusion across the barrier, because oxygen is rapidly transferred from maternal to fetal blood due to the high permeability of the placental barrier to respiratory gases. Although direct evidence for this assumption in the human is lacking, experimental support for flow limitation of maternal–fetal oxygen transfer is observed in sheep (reviewed in Ref. 154). Collectively, these studies demonstrated that umbilical oxygen delivery was linearly related to blood flow in response to acute reductions in either umbilical or uterine blood flow. Importantly, the fetus maintained oxygen consumption by increasing fetal fractional oxygen extraction, resulting in a lower umbilical arterial pO_2. This compensatory mechanism is of major physiological importance during labor and physical exercise, where short periods of reduced blood flow reduction are common. In response to chronic blood flow reduction, however, fetal sheep were not able to maintain normal oxygen consumption, which was associated with decreased fetal growth rate.[154] Because O_2 transfer is flow limited, the geometry of placental blood flow influences the efficiency of transport (reviewed in Refs 156,157). The types of geometric arrangement of blood flow on either side of the placental barrier, as discussed in this chapter, may explain differences in oxygen transfer efficiencies among species.

Net transfer of oxygen in the maternal–fetal direction is facilitated by a marked difference in pO_2 between maternal and fetal blood. This difference is large when comparing pO_2 in maternal arterial blood (100 mmHg) and in the umbilical vein (approximately 34–41 mmHg at the end of pregnancy).[158,159] This large pO_2 gradient cannot be attributed to restriction of oxygen diffusion across the placental barrier, but is likely to be due to the mixing of "arterial" and "venous" blood in the intervillous space, resulting in a pO_2 at the maternal side of the exchange barrier that is much lower than in maternal arterial blood. Furthermore, it has been estimated that as much as 40% of uterine oxygen uptake is consumed by the placenta itself,[160] which further contributes to the maternal–fetal gradient. Other factors that facilitate the movement of oxygen in the maternal–fetal direction are higher hemoglobin concentrations in fetal blood, resulting in a significantly higher oxygen carrying capacity, and a higher affinity of fetal hemoglobin (HbF) for oxygen compared to maternal hemoglobin (HbA). The higher oxygen affinity of HbF is due to its lower affinity to 2,3-diphosphoglycerate, causing a left shift in the oxygen–hemoglobin dissociation curve.

Many vascular beds (such as the brain, muscle, or kidney) are characterized by extensive local autoregulation, which can modify blood flow in response to changes in either oxygen delivery or demand. There is little evidence supporting significant autoregulation or reactive hyperemia in the placental circulation. Short-term regulation of placental oxygen transfer is therefore limited to changes in fetal oxygen extraction and, to some extent,

decreased affinity of hemoglobin for oxygen when CO_2 is bound (the Bohr effect). Long-term regulation involves increased hematocrit (polycythemia) in fetal blood in response to hypoxemia, which will increase the oxygen carrying capacity. However, increased red blood cell mass may result in elevated viscosity, increased peripheral resistance, and decreased blood flow, curtailing any advantages with increased erythrocyte volume.

Acid–Base: Carbon Dioxide, Protons, and Lactate

The fetus relies on the placenta for regulation of its acid–base balance. This delicate balance is mainly dependent upon the exchange of carbon dioxide, protons, and lactate across the placental barrier (Figure 39.11).

Carbon Dioxide

The fetus and the placenta continuously produce large amounts of carbon dioxide as a result of oxidative metabolism. In postnatal life, CO_2 is readily eliminated via the lungs. It is also in equilibrium with carbonic acid, which can rapidly produce protons. In the context of fetal acid–base balance, CO_2 is characterized as a "respiratory acid". Bicarbonate transporters are unlikely to have a major role in placental CO_2 elimination, which primarily takes place as diffusion of dissolved gas. Carbon dioxide is highly soluble in lipids. Transplacental transfer of CO_2 is therefore believed to be blood flow limited. Due to mild physiological hyperventilation of pregnancy, arterial partial pressure of carbon dioxide (pCO_2) is lower in pregnant women (26–34 mmHg) when compared to nonpregnant women (38–42 mmHg). Umbilical vein pCO_2 at term ranges from 38 to 45 mmHg, providing a substantial diffusion gradient that promotes fetal–maternal CO_2 transfer. Blood CO_2 is predominantly present as H^+ and HCO_3^- ions. Some CO_2 is associated with deoxygenated Hb, as carbamino–Hb, and only a small amount is dissolved. In the placenta, CO_2 is eliminated across the placental barrier, and this process is facilitated by conversion of H^+ and HCO_3^- to CO_2, a reaction catalyzed by the action of carbonic anhydrase expressed in erythrocytes, endothelial and trophoblast cells. Reduced placental and/or umbilical blood flow may cause respiratory acidosis, which is normalized following the reestablishment of adequate blood flows. Oxygenation influences placental CO_2 transfer because binding of oxygen decreases the affinity of hemoglobin to bind CO_2.

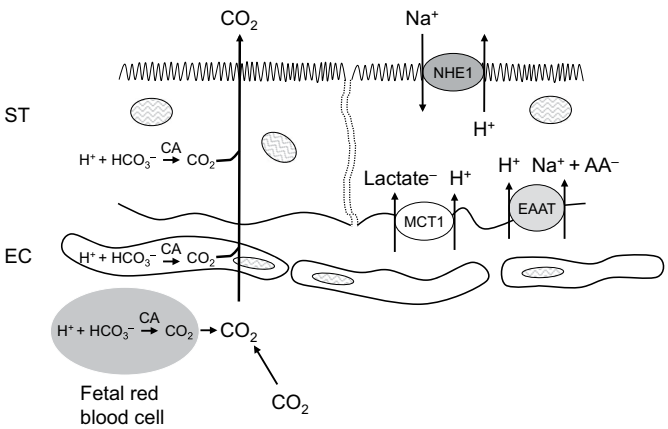

FIGURE 39.11 The role of the placenta in fetal pH regulation: transport of CO_2 and protons. Carbon dioxide readily diffuses across the placental barrier as dissolved gas. Metabolically produced protons are eliminated across the placenta, mediated by H^+–lactate co-transport in the BM, which is, in turn, mediated by MCT1, followed by extrusion of protons in exchange for sodium across the MVM and mediated by the sodium–proton exchanger (NHE). It is also possible that excitatory amino acid transporters (EAAT), which are known to co-transport Na^+, H^+, and anionic amino acids, contribute to the transfer of protons across the BM. MCT1: monocarboxylate transporter 1; CA: carbonic anhydrase; ST: syncytiotrophoblast; EC: endothelial cell.

Protons

Acid equivalents produced by fetal metabolism cannot be eliminated by transfer of CO_2 across the placenta, but require movement of protons from the fetal to the maternal circulation. Although transport of bicarbonate in the opposite direction could alleviate a proton load, a transport mechanism mediating bicarbonate transfer in the maternal-to-fetal direction remains unknown. The sodium–proton exchanger (NHE) is the most important placental transporter involved in acid–base regulation. There are at least nine distinct human NHE genes (NHE1/SLC9A1–NHE9/SLC9A9, where SLC9A9 is solute carrier family 9, subfamily A, member 1). This family of transporters is involved in a host of physiological processes that include not only the removal of intracellular protons in exchange for sodium but also the regulation of cell volume, proliferation, and apoptosis. NHEs are highly active in the MVM,[161] mediating the export of protons from the syncytial cytosol into maternal blood (Figure 39.11). Studies in villous fragments ex vivo have confirmed that NHE is critical for maintenance of intracellular pH in the syncytiotrophoblast.[162] Pharmacological[163] as well as protein expression data[164,165] suggest that NHE1 is the major isoform expressed in the MVM, with lower expression of NHE2 and NHE3.[164] NHE activity has been reported to be absent from the BM,[166] consistent with a very low expression of NHE1–3 in this membrane.[164] Studies in term villous fragments have demonstrated that placental NHE is activated by epidermal growth factor, sphingosine-1-phosphate, aldosterone, and cortisol.[167,168] Estrogen is known to decrease placental NHE3 expression and activity in the baboon.[169]

Lactate

Lactate is an important energy source in the fetal heart, brain, and skeletal muscle. Fetal levels of lactate

TABLE 39.1 Glucose Transporter Isoform Protein Expression in the Human Placenta

Isoform	Localization	References	Remark
GLUT1 (SLC2A1)	*First trimester*: MVM, CT *At term*: MVM, BM, EC, CT	175–177	Particularly high expression in ST plasma membranes with MVM expression threefold higher than BM
GLUT3 (SLC2A3)	*First trimester*: MVM, CT *At term*: MVM?, EC	177–179	High-affinity (low K_m) isoform, highly expressed in the brain
GLUT4 (SLC2A4)	*First trimester*: MVM *At term*: SC	177,180	Insulin-sensitive glucose transporter
GLUT9 (SLC2A9)	*At term*: MVM (GLUT9b), BM (GLUT9a)	181	Transports glucose, fructose, and urate with high affinity
GLUT12 (SLC2A12)	*First trimester*: ST *At term*: SC, VSM	182	Insulin-sensitive glucose transporter

MVM: microvillous plasma membrane; BM: basal plasma membrane; EC: endothelial cell; CT: cytotrophoblast; ST: syncytiotrophoblast; SC: stromal cells; VSM: vascular smooth muscle.

are higher than maternal levels. The placenta may be a source of lactate even when oxygen supplies are adequate. However, the placenta is also an important site for clearance of fetal lactate, especially in cases of fetal hypoxia. Lactate is transported across cell membranes by members of the monocarboxylate transporter (MCT) family (SLC16), which mediate H^+–lactate co-transport. Both the MVM[170] and BM[170] efficiently transport lactate in the presence of a proton gradient. The predominant MCT isoform in the MVM is MCT4, whereas MCT1 is the main form in the BM.[170]

It is believed that the primary route for placental elimination of metabolically produced protons by the fetus involves H^+–lactate co-transport across the BM, followed by extrusion of protons in NHE-based exchange for sodium across the MVM (Figure 39.11). It is also possible that excitatory amino acid transporters, which are active in the BM,[171] contribute to the transport of protons from the fetal compartment into the syncytiotrophoblast across the BM. These transporters mediate the cellular uptake of anionic amino acids, coupled to the co-transport of Na^+ and H^+.

Sodium, Potassium, Iodide, Chloride, and Bicarbonate

Sodium

Sodium is the predominant extracellular cation. The osmotic pressure produced by this ion is an important determinant of extracellular volume. Sodium transport across epithelia typically involves passive entry across the apical membrane by several mechanisms, and an active efflux step across the basolateral membrane mediated by Na^+K^+–ATPase. Placental sodium transport fits this general model, with several unique characteristics. A large number of Na^+-dependent co-transporters are active in the MVM. These transporters utilize the inwardly directed sodium gradient across the MVM to energize the co-transport of amino acids (Table 39.2), phosphate, biotin, and succinate, or the efflux of molecules such as protons via exchange mechanisms. Akin to other cells, low intracellular sodium concentrations in the syncytiotrophoblast are maintained by Na^+K^+–ATPase. However, unlike typical epithelia in which the sodium pump is expressed exclusively in the basolateral plasma membrane, Na^+K^+–ATPase is expressed and active in both the BM and MVM[174] of human syncytiotrophoblasts. It has been proposed that excessive Na^+ efflux across the BM may result in overhydration of the fetal compartment, resulting in elevated blood pressure or polyhydramnios.[174] Therefore, the sodium gradient that drives nutrient and ion uptake and waste removal is counterbalanced by an efficient Na^+K^+–ATPase-dependent, MVM-based mechanism that returns sodium to the maternal compartment and maintains the gradient. Although this model remains hypothetical in the human placenta, there is significant experimental support for the presence of a similar mechanism in the rat placenta.[132]

Potassium

Potassium is the main intracellular cation. Na^+K^+–ATPase constitutes the primary pathway by which K^+ is taken up into many cells, including syncytiotrophoblasts. Potassium is believed to exit the syncytium passively through a large number of different potassium channels that are present in the syncytiotrophoblast membranes,[198,199] driven by the large outwardly directed electric and concentration gradient. It is unclear which of these channels participate in transplacental K^+ transport across the MVM and BM.

Chloride

Chloride is the main extracellular anion. It is transported across plasma membranes in a process mediated by multiple mechanisms, including exchange with bicarbonate or through channels. Chloride uptake is also

TABLE 39.2 Amino Acid Transport Systems in the Human Placenta at Term

System	Activity	Protein	Gene	Protein Localization	Substrates	References
NA^+-DEPENDENT TRANSPORTERS FOR NEUTRAL AMINO ACIDS						
A	MVM ≫ BM	SNAT1	SLC38A1	MVM	(Gly), Ala, Ser, Cys, Gln, Asn, His, (Met), MeAIB	183 184–186
		SNAT2	SLC38A2	MVM	Gly, Pro, Ala, Ser, Cys, Gln, Asn, His, Met, MeAIB	
		SNAT4	SLC38A4	MVM, BM	Gly, (Pro), Ala, Ser, Cys, Asn, (Met), (MeAIB)	
ASC	BM MVM?	ASCT1	SLC1A4		Ala, Ser, Cys	185,186
		ASCT2	SLC1A5		Ala, Ser, Cys, Thr, Gln	
B^0	? mRNA highly expressed	B^0AT1	SLC6A19	nd	Ala, Asn, Cys, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	
		B^0AT2	SLC6A19	nd		
N	MVM?	SNAT3	SLC38A5	nd	His, Gln, Asn	187,188
		SNAT5	SLC39A3	nd		
Gly	MVM	XT2	SLC8A18	nd	Gly	189
β	MVM ≫ BM	TAUT	SLC6A6	MVM, BM	Tau, β-ala	190,191
NA^+-INDEPENDENT TRANSPORTERS FOR NEUTRAL AMINO ACIDS						
L	MVM, BM	LAT1	SLC7A5	MVM	(Gln), His, Met, Leu, Iso, Val, Phe, Tyr, Trp, BCH	185,186,192
		LAT2	SLC7A8	MVM (50 kD) BM (30 kD)	Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Leu, Iso, Val, Phe, Tyr, Trp	
	BM?	LAT4	SLC43A2	nd mRNA expressed	Phe, Leu, Iso, met, BCH	193
TRANSPORTERS FOR CATIONIC AMINO ACIDS						
y+	MVM>BM	CAT1	SLC7A1	BM	Arg, lys, His	194,195
		CAT2B	SLC7A2	nd	Arg, lys, His	
		CAT3	SLC7A3P	nd	Arg, lys	
		CAT4	SLC7A4	nd	Unknown	
y+L	BM>MVM	y+LAT1	SLC7A7	nd	Lys, Arg, Gln, His, Met, Leu[a]	194–196
		y+LAT2	SLC7A6	nd	Lys, Arg, Gln, His, met, Leu,[a] Ala, Cys	
$b^{0,+}$	BM?	$b^{0,+}$ AT	SLC7A9	nd	Lys, Arg, Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Iso, Leu, Val, Phe, Tyr, Trp	195,196
TRANSPORTERS FOR ANIONIC AMINO ACIDS						
X^-_{AG}	MVM, BM	EAAT1	SLC1A3	nd	Glu, Asp, Cys	171,197
		EAAT2	SLC1A2	nd	Glu, Asp	
		EAAT3	SLC1A1	nd	Glu, Asp	

ST: syncytiotrophoblast; MVM: microvillous plasma membrane; BM: basal plasma membrane; nd: not determined.
[a]*Na^+-dependent influx, but not efflux.*

mediated by co-transport with Na^+ and can leave cells by co-transport with K^+. A wide range of chloride transporters and channels are expressed in the MVM. Far less information exists regarding chloride transport through the BM. The anion exchanger 1 (AE1, SLC4A1) is present in the MVM throughout gestation[200–202] but not in the BM.[201] Electrophysiological studies using isolated human villi, as well as experiments with reconstituted liposomes, have provided evidence for a high-conductance ("maxi") chloride channel in the MVM.[203,204] Intermediate diphenylamine-2-carboxylic acid (DPC inhibitable) and small protein kinase A–ATP–cAMP-activated chloride channels have also been identified in the MVM. Additional MVM chloride channels include CLI5, cystic fibrosis transmembrane regulator, and multidrug resistance protein 1 (MDR1) P-glycoprotein. The physiological roles and mechanism of action of these chloride transporters and channels remain unclear. It is highly unlikely that net transplacental chloride transport is mediated by passive transport via transtrophoblastic channels, because chloride concentrations are 5 mM higher on the fetal side of the barrier, fetal side is slightly negative,[132] and the direction of the hydrostatic pressure gradient in the human placenta in vivo is in the fetal-to-maternal direction.[136,137] Thus, the concentration and electrical gradients as well as hydrodynamic water flow toward the mother through the transtrophoblastic channels would all favor passive chloride movement in the fetal–maternal direction.

Bicarbonate

Bicarbonate transporters are members of the SLC4 family as well as the SLC26 SLCA family of sulfate transporters, some of which also transport bicarbonate. Based on protein expression and functional data, AE1 (SLC4A1), a transporter that exchanges chloride for bicarbonate is present in the MVM[200,201] but not in the BM.[201] Given the intracellular and extracellular concentrations of chloride and bicarbonate and the MVM localization of AE1, this transporter is predicted to mediate chloride entry into the syncytium and bicarbonate efflux into the maternal blood. However, the physiological function of transport of bicarbonate to the maternal circulation is unclear.

Iodide

Iodide transfer from the mother across the placenta is essential for fetal thyroid hormone production, and likely begins early in the second trimester. The mechanisms responsible for placental iodide transport remain to be fully established, but likely involve uptake across the MVM, mediated by the sodium iodide symporter (NIS, also known as SLC5A5), which is expressed in the placental MVM[205] and in cultured trophoblasts.[205] Pendrin (SLC26A4), a Cl^-–OH^-–HCO_3^- exchanger that also transports iodide, is expressed in the MVM[205]; however, the role of this transporter in maternal–fetal iodide transfer is unclear because pendrin mediates iodide efflux in other tissues. Recently, it was reported that the human Na^+–multivitamin transporter (hSMVT, also known as SLC5A6), which is expressed in the placenta, mediates cellular uptake of iodide,[206] but the contribution of hSMVT to placental iodide transport is currently unknown.

Water

Near term, the human fetus accumulates 22 ml of water per day. Because only approximately 20% of this can be accounted for by metabolically produced water, the large fraction of fetal water accretion must be transferred from the maternal circulation, predominantly transported across the placenta.[132] The driving forces for net maternal–fetal water transfer, and the pathways for water and solute transfer across the placental barrier in the human, are not fully defined. Existing information regarding hydrostatic pressure in umbilical circulation and intervillous space[136,137] suggests a higher hydrostatic pressure in the fetal side of the placenta, which would promote transfer of water from the fetus to the mother. Because differences in colloidal osmotic pressure[132] also promote water movement toward the mother, net transplacental movement of water must be driven by a gradient of solutes. However, no significant gradient has been clearly demonstrated when maternal and fetal osmotic pressures are measured. This apparent contradiction may be reconciled by studies demonstrating that the syncytiotrophoblast plasma membranes have significant water permeability.[207] Thus, given the large surface area of the placental barrier, only a very small solute gradient is needed to account for net fetal water accumulation. It is therefore possible that water accumulation by the human fetus could be driven by a solute gradient across the placental barrier that is small enough to be within the margin of error of standard osmolarity measurements.[207] Available data from experiments using the human placenta suggest that solutes that are actively transported into the fetal compartment generate a small osmotic gradient, which causes osmotic water movement via a transcellular pathway. Subsequently, water recirculates back to the maternal compartment through transtrophoblastic channels driven by the hydrostatic and colloid osmotic pressure gradients, preventing overhydration of the developing fetus. A similar model has been proposed for the rat placenta.[132]

The aquaporins (AQPs) are a family of membrane water channels.[208] Cells expressing aquaporins in the plasma membrane have a 2- to 50-fold higher permeability to water than cells lacking water channels. There are 13 mammalian AQP isoforms, some of which (e.g., AQP3, AQP8, and AQP9) also transport glycerol. In addition, AQP9 has been shown to transport other polar solutes, including

amino acids and sugars.[209] AQP8 transports ammonium as well as water.[210] Replace by AQP3, AQP8, and AQP9, and/or protein are expressed in the human syncytiotrophoblast.[211,212] However, the role of these aquaporins in the syncytiotrophoblast remains to be fully established, and evidence to suggest significant AQP-mediated water permeation across isolated microvillous and basal plasma membranes is lacking.[207]

Calcium

During pregnancy, the fetus accumulates approximately 30 g of calcium, predominantly in the third trimester, when fetal bone mineralization occurs. As in other cells, the intracellular calcium concentration in the syncytiotrophoblast is four magnitudes lower than extracellular concentrations, which is critical for the function of intracellular calcium as a signal transducer.

In principle, the transfer of calcium across the syncytiotrophoblast occurs in three distinct steps (reviewed in Ref. 213) (Figure 39.12) similar to calcium transport in the intestinal epithelium. The first step involves calcium entry across the MVM into the cell. This is followed by Ca^{2+} mobilization through the cytosol in association with calcium-binding proteins. The third step, calcium is transported out of the cell, in a process mediated by Ca^{2+} ATPase. Members of the transient receptor potential (TRP) gene family, specifically TRPV5 and TRPV6, are calcium-selective channels serving as apical calcium entry pathways in absorptive and secretory tissues. Of these channels, TRPV6 is highly expressed in the placenta and is functional in cultured primary human

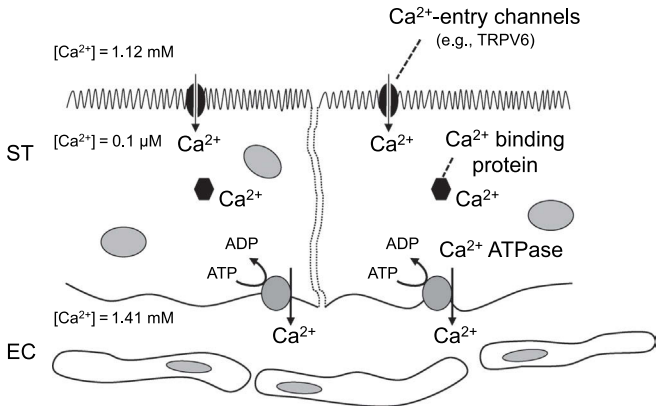

FIGURE 39.12 Placental calcium transport. Calcium is believed to enter the syncytiotrophoblast across the MVM via Ca^{2+}-entry channels such as TRPV6. In the cytoplasm, calcium is rapidly sequestered into intracellular compartments or bound to calcium-binding proteins, such as calmodulin or calbindin, which transport calcium through the cytoplasm and buffer intracellular calcium. The plasma membrane Ca^{2+} ATPase, which is highly expressed and functional in the BM, is the primary mechanism for Ca^{2+} efflux out of the syncytiotrophoblast into the fetal circulation. TRPV6: transient receptor potential cation channel, subfamily V, member 6; ST: syncytiotrophoblast; EC: endothelial cell.

trophoblasts.[214] Store-operated channels (SOC), which are calcium channels in the plasma membrane that are activated by depletion of intracellular calcium stores, are expressed and are functional in the syncytiotrophoblast of term villous fragments,[215] potentially representing a pathway for calcium entry across the MVM. Furthermore, ATP increases calcium influx into trophoblasts in a process mediated by interaction with the P2 purinergic receptor family.[216] Several studies showed the presence of L-type voltage-dependent calcium channels (VDCCs) in trophoblasts, particularly in the MVM.[217–219] However, the specific physiological function of these channels is poorly understood, because Ca^{2+} uptake in the MVM is insensitive to L-type calcium channel blockers.[220] It has been suggested that MVM L-type calcium channels are involved in cellular signaling and regulation of protein secretion from trophoblasts.[213] In addition to these calcium-selective channels, numerous nonselective cation channels have been identified in the MVM, such as polycystin-2,[221] which also may contribute to Ca^{2+} entry into syncytiotrophoblasts. TRPV5, TRPV6, and L-type VDCC have been shown to be expressed and functional in the BM[222]; however, the function of these BM channels remains to be established.

Once calcium enters the cytoplasm, it is rapidly sequestered into intracellular compartments, or is bound to calcium-binding proteins such as calmodulin or calbindin. Sequestration into the endoplasmic reticulum allows for regulation of intracellular calcium concentrations and release of calcium in response to signaling through the diacylglycerol–protein kinase C–inositol P_3 pathway. Cytoplasmic calcium-binding proteins transport calcium through the cytoplasm and buffer changes in intracellular calcium. Studies in the rat placenta showed that calbindin-D_{9k} is highly expressed in late gestation, where it is the rate-limiting step in transplacental transport.[223] However, reports remain conflicting with respect to the expression of calbindin-D_{9k} in the human placenta.[224,225]

There are two principal mechanisms by which Ca^{2+} is actively transported out of the cell against a significant electrochemical gradient: the Na^+–Ca^{2+} exchanger and the calcium pump (or Ca^{2+} ATPase). Whether the Na^+–Ca^{2+} exchanger is expressed in the syncytiotrophoblast remains unclear.[226,227] Functional studies indicate that Na^+–Ca^{2+} exchange does not play a major role in Ca^{2+} efflux in syncytiotrophoblasts.[228] The plasma membrane Ca^{2+} ATPase (PMCA), on the other hand, is highly expressed and functional in the BM[226,229] and constitutes the predominant mechanism for the maintenance of low free intracellular calcium concentrations in the syncytium. Notably, there are four isoforms of PMCA (PMCA1–4). PMCA1 and PMCA4 are ubiquitous, including in the syncytiotrophoblast BM.[229] The activity of the BM calcium pump significantly increases from gestational week 32 through 37, suggesting an increased

transport capacity during the period of maximal fetal skeletal mineralization.[229]

Our understanding of the mechanisms regulating placental calcium transport is incomplete. The influence of the major calcitropic hormones, parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxycholecalciferol (vitamin D), on placental calcium transport has been studied mainly in animal models (reviewed in Ref. 132), and revealed that vitamin D stimulates and calcitonin inhibits placental calcium transport in the sheep, but not in the rat, placenta. Evidence also suggests that parathyroid hormone–related peptide (PTHrP) is the primary hormonal regulator of placental calcium transport. PTHrP is synthesized by several fetal tissues, such as the parathyroid glands, as a 141-amino-acid pro-hormone. It is subsequently cleaved into several fragments of smaller molecular weight. Synthetic PTHrP midmolecule fragments (67–86 or 38–94) have been shown to maintain the calcium gradient across the sheep placenta.[230] In mice with homozygous deletion of the PTHrP gene, placental transport of calcium is decreased and the normal Ca^{2+} concentration gradient across the placental barrier (fetal Ca^{2+} concentrations > maternal) fails to develop. Transplacental calcium transport increased in PTHrP-deficient fetuses by administration of PTHrP (1–86) or PTHrP (67–86) to the fetus, but not PTHrP (1–34) or intact PTH.[231] These studies suggest that the midmolecular region of PTHrP plays a critical role in stimulating the placental transport of calcium, at least in some species. Human placental Ca^{2+} ATPase can be activated by PTHrP (38–94),[232] consistent with the possibility that fetal PTHrP regulates placental calcium transport also in pregnant women.

Immunoglobulins

Among all subclasses of immunoglobulins, only IgG is transferred at a significant level across the human placenta. The levels of IgG in the first and early second trimesters of pregnancy are low, with increased transport in the second half of pregnancy, which continue to gradually increase until term.[233,234] The rate of IgG transport is subtype specific, with a declining order of transport rate for IgG1, IgG4, IgG3, and IgG2. At term, the level of IgG1 in the fetal blood exceeds that of maternal blood.[233,235]

A first step in IgG transport is binding of the Fcγ component of IgG to the syncytiotrophoblast, with subsequent transport of the whole molecule within a coated vesicle.[236] The human neonatal Fcg receptor (FcRn), which is homologous to major histocompatibility complex class I, is expressed in the syncytiotrophoblast and mediates IgG uptake into the placenta.[237,238] FcRn exhibits a pH-dependent interaction with IgG, exhibiting high affinity to IgG at lower pH and low affinity at high pH. It is thus assumed that binding to FcRn does not take place at the blood facing the trophoblastic microvillous membrane, where the pH is neutral, but within the acidic environment of trophoblastic endosomes, which protects the IgG against degradation.[237,239] Following the transfer of IgG across the syncytiotrophoblast, it is either transported via the basal membrane to the stroma, where it binds Fcγ receptors that are expressed in Hofbauer and stromal cells,[237] or transferred across the fetal capillary endothelial cells into the fetal circulation. Fetal endothelial cells express FcγRIIb2, but its function is not entirely clear.[240] Lastly, it is noted that uptake of IgG in rodents likely occurs mainly via the yolk sac, which exhibits a high expression of Fc receptors.[241]

Glucose

Glucose is the primary substrate for energy metabolism in the placenta and fetus. In the absence of significant fetal gluconeogenesis, the fetus is critically dependent on transplacental glucose transfer. Of the total amount of glucose taken up by the placenta from the uterine circulation, 30–40% is consumed by the placenta itself. Classical studies by Widdas demonstrated that transplacental glucose transport in the sheep is mediated by facilitated diffusion.[242] Numerous studies have subsequently confirmed that placental glucose transport is facilitated, Na^+ independent, saturable, stereospecific, and selective

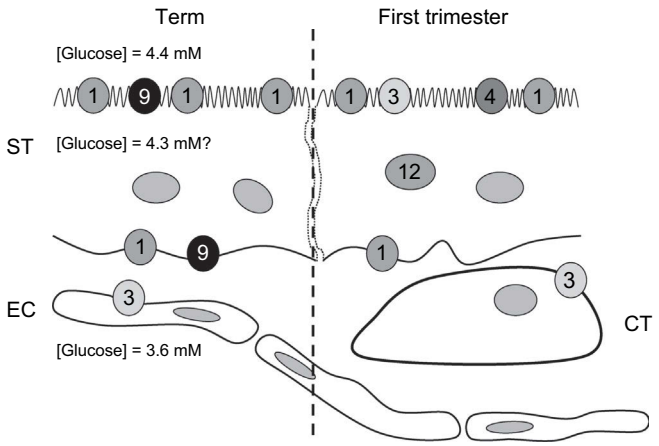

FIGURE 39.13 Placental glucose transport. Glucose transfer across the human placenta is facilitated by glucose transporters (GLUTs; isoforms indicated by numbers) expressed in the two polarized syncytiotrophoblast plasma membranes. At term (left panel), GLUT1 is the primary glucose transporter in the placental barrier, with threefold higher expression in the MVM than in the BM. Transport across the BM is believed to be the rate-limiting step in transplacental glucose transfer. GLUT9 is also expressed in the MVM and BM; however, the functional importance of this transporter in the placenta remains to be established. In the first trimester (right panel), at least four different GLUT isoforms are expressed in the syncytiotrophoblast: GLUT1, GLUT3, GLUT4, and GLUT12, of which GLUT4 and GLUT12 are sensitive to regulation by insulin. ST: syncytiotrophoblast; EC: endothelial cell.

in all species studied (reviewed in Ref. 243). The human placenta is no exception, because glucose transfer is facilitated by glucose transporters expressed in the two polarized syncytiotrophoblast plasma membranes (Figure 39.13).[175,244,245] Consistent with the facilitated nature of the transport process, fetal plasma glucose concentrations are lower than maternal levels.[246] However, given the high placental glucose consumption, the maternal–fetal concentration difference (less than 1 mM) is remarkably small, illustrating the high capacity of the placental glucose transport system.

The facilitative glucose transporter family (glucose transporter (GLUT) also known as SLC2) includes 14 isoforms that share common structural features, such as 12 transmembrane domains, both N and C termini facing the interior of the cell, and an extracellular *N*-glycosylation site. Many of the GLUT isoforms are expressed in the human placenta (Figure 39.13 and Table 39.2); however, only a few of these are likely to be important in mediating maternal–fetal glucose transport.

GLUT1 (SLC2A1) was the first GLUT isoform to be cloned and characterized and is ubiquitously expressed. GLUT1 protein is expressed in most placental cells, including the syncytium, cytotrophoblasts, and endothelial cells.[175–177] GLUT1 expression is particularly high in the syncytiotrophoblast plasma membranes, with threefold higher expression in the MVM compared to the BM,[175] reflecting the higher glucose transport activity in the MVM.[175] Given the six- to sevenfold larger area of the microvillous surface of the syncytiotrophoblast compared to the BM at term, it has been estimated that the total glucose transport capacity of the MVM may be 20-fold higher than that of the BM.[175] As a result, the glucose concentration within the syncytiotrophoblast may be maintained close to glucose concentrations in the intervillous space, providing a maximal gradient for transfer to the fetus.[175] Based on these findings, it was suggested that transport across the BM constitutes the rate-limiting step of transplacental glucose transport.[175] Alternatively, glucose transporter abundance in the placental barrier may have a lesser impact on transplacental glucose transport, because glucose transfer is highly dependent upon placental and umbilical blood flow.[247] Maternal–fetal glucose transport is likely to be influenced by both of these factors. With all other factors kept constant, any alteration in glucose transporter expression or activity in the BM is likely to alter glucose flux across the barrier, a concept that is supported by studies in polarized BeWo cells[248] and mathematical modeling of glucose transport in the human placenta.[249]

GLUT3 (SLC2A3) is a high-affinity glucose transporter, which was considered to be neuron specific. However, GLUT3 protein is also expressed in other cells and tissues with a high glucose demand, in particular in spermatozoa, preimplantation embryos, and certain cancer cells. In the first trimester, GLUT3 protein is expressed in cytotrophoblast cells[177] and in the MVM.[177,178] GLUT3 protein is also expressed in fetal capillary endothelium in the term placenta.[179] Although GLUT3 mRNA is present in term trophoblast,[179] studies of trophoblast GLUT3 protein expression in the MVM have generated conflicting results.[175,178,179] GLUT4 (SLC2A4) is of major physiological importance because of its critical role in insulin-stimulated glucose uptake in muscle and adipose tissue. Whereas GLUT4 protein is expressed in the cytoplasm and MVM of first-trimester syncytiotrophoblasts,[177] at term the transporter is expressed in stromal cells but not in trophoblasts.[180] GLUT9 (SLC2A9) is a high-affinity transporter for glucose, fructose, and urate. Alternative splicing results in two proteins, GLUT9a and GLUT9b, which differ in their N terminal. These isoforms appear to determine transporter trafficking to apical or basolateral domains in polarized epithelia. GLUT9 is highly expressed in the kidney and liver, and was recently shown to be present in the human placenta, with GLUT9a predominantly expressed in the BM and GLUT9b in the MVM.[181] GLUT12 is an insulin-sensitive isoform that is mainly expressed in muscle, small intestine, and prostate. GLUT12 is also expressed in the syncytiotrophoblast in early pregnancy and in the villous stroma at term.[182]

There are striking differences in glucose transporter expression in syncytiotrophoblasts between the first trimester and term (Figure 39.13). In the first trimester, at least four different GLUT isoforms are expressed in the syncytiotrophoblast: GLUT1, GLUT3, GLUT4, and GLUT12,[177,182] of which GLUT4 and GLUT12 are sensitive to regulation by insulin. However, in late pregnancy GLUT1 is the primary GLUT isoform mediating glucose transport across the placental barrier in the human.[175,177] These differences may explain experimental findings that insulin stimulates placental glucose uptake primarily in the first trimester.[177] It is possible that the expression of insulin-sensitive glucose transporters in the first-trimester syncytium is critical for regulation of glucose transport and support of feto-placenta growth in early pregnancy, prior to the establishment of blood flow in the intervillous space after 12 weeks of gestation. In addition, first-trimester fetal physiology is characterized by anaerobic metabolism and therefore a high demand for glucose, which may require a different set of transporters. Thus, the high expression of insulin-sensitive glucose transporters in the first-trimester MVM may be necessary in order to meet the relatively high glucose requirements of the developing embryo.[177]

Because the GLUT1 transporters are not saturated at normal blood glucose levels, net maternal–fetal glucose transfer is also dependent on the glucose concentration difference between maternal and fetal blood. Insulin regulation of placental glucose transport at term

remains controversial, with some studies showing that insulin increases placental glucose uptake, whereas other investigators have reported no effect.[250] In a first-trimester trophoblast cell line, glucose transport activity was stimulated by insulin, IGF-I, and IGF-II.[251,252] Placental glucose transporter activity was not affected by hormones such as leptin, GH, IGF-I, insulin, and cortisol in primary villous fragments from term placentas.[253] Glucocorticoids are known to decrease placental glucose transporter expression. Corticotrophin-releasing hormone (CRH) increased GLUT1 and decreased GLUT3 mRNA and protein expression in cultured primary human trophoblasts isolated from term placentas.[254] Because CRH is produced locally in the placenta, these findings are consistent with autocrine–paracrine regulation of placental glucose transport by CRH. Increased oxidative stress in placental explants causes reduced glucose uptake as well as lowered expression of GLUT1. Chronic hypoxia in vivo, as in high-altitude pregnancies, also reduces the expression of GLUT1, but the effect is restricted to the BM and not in the MVM.

Amino Acids

Amino acids are required for fetal protein synthesis, and they constitute critical precursors in many biosynthetic pathways, including the synthesis of porphyrins, nitric oxide, neurotransmitters, and nucleotides. Nonessential amino acids are also used as fetal energy substrates. It is estimated that 32% of the energy requirement of well-nourished fetal sheep is met by amino acid oxidation.[255] For most amino acids, the concentrations in the umbilical vein are two- to threefold higher than in the uterine vein, demonstrating that the transfer of amino acids across the placental barrier is an active process (reviewed in Refs 256–258). This is also supported by the observation that placental concentrations of amino acids are, in general, much higher than in the blood on either side of the placental barrier (Figure 39.14).

There are two main types of amino acid transporters—accumulative transporters and amino acid exchangers—both of which are found in the MVM. Accumulative transporters mediate cellular uptake, resulting in increased intracellular amino acid concentrations. Amino acid exchangers, in contrast, exchange one amino acid for another, resulting in altered amino acid composition without changing total concentration.[259] The primary driving forces for amino acid uptake across the MVM mediated by accumulative transporters are (1) the inwardly directed Na^+ gradient (e.g., the System A transporter and the taurine transporter), and (2) the potential difference between the intracellular and extracellular environments, which is the driving force for uptake of the cationic amino acids (arginine, histidine, and lysine). The L-amino acid transporter system is an example of

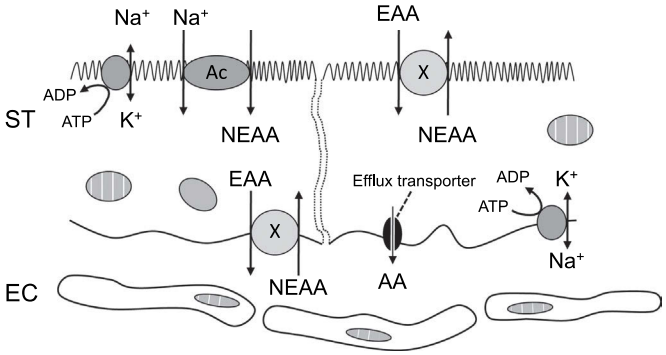

FIGURE 39.14 A model for placental amino acid transport. The uptake of amino acids from the maternal circulation across the MVM into the syncytiotrophoblast represents the active step of amino acid transport and is mediated by accumulative transporters (Ac) and amino acid exchangers (X). Accumulative transporters mediate cellular uptake, resulting in increased intracellular amino acid concentrations. Amino acid exchangers, on the other hand, exchange one amino acid for another, resulting in altered amino acid composition without changing the total concentration. The driving forces for the amino acid uptake mediated by accumulative transporters are the inwardly directed Na^+ gradient, or the potential difference with the inside of the cell negative. Exchangers, such as System L, use the steep, outwardly directed concentration gradient of some nonessential amino acids (NEAA) to drive the uptake of essential amino acids (EAA) against their concentration gradients. In all of these cases, the energy for the uphill transport is ultimately generated by the Na^+K^+–ATPase. Amino acids are transferred across the BM by facilitated diffusion driven by the outwardly directed concentration gradient mediated by exchangers and efflux transporters. ST: syncytiotrophoblast; EC: endothelial cell.

an exchanger, which uses the steep outwardly directed concentration gradient of some nonessential amino acids to drive the uptake of essential amino acids, such as leucine, against its concentration gradient. The energy for the uphill transport of amino acids is ultimately generated by the Na^+K^+–ATPase, which extrudes sodium in exchange for potassium, thereby maintaining a low intracellular Na^+ concentration and creating a potential difference across the plasma membrane.[174] Subsequently, amino acids are transferred across the BM by facilitated diffusion driven by the outwardly directed concentration gradient (Figure 39.14). While accumulative transporters and exchangers are sufficient to account for the uptake of all amino acids in most cells, in transporting epithelia, such as the syncytiotrophoblast, efflux transport mechanisms in the BM are needed to allow net transfer of all amino acids across the epithelium.[258]

The asymmetric distribution of amino acid transporters between the syncytiotrophoblast MVM and BM is critical in order to generate a net flux of amino acids from mother to fetus (Figure 39.14). The human syncytiotrophoblast expresses at least 20 different amino acid transporters (Table 39.2), with each transporter mediating the uptake of several amino acids, and each amino acid can be transported by multiple transport systems. Specifically, accumulative transporters and amino acid exchangers are

present in the MVM, whereas amino acid exchangers and efflux transporters predominate in the BM. Figure 39.14 represents a simplified depiction of placental amino acid transport pathways. Notably, it does not take into account placental amino acid metabolism or complex interactions among transporters. A series of studies of amino acid metabolism in the sheep fetus revealed that there is an intricate cycling of amino acids between the placenta and the fetal liver (reviewed in Refs 260,261). Specifically, glutamate is taken up from the umbilical, rather than the uteroplacental circulation, and is oxidized or converted to glutamine. Glutamine is released from the placenta into the fetal circulation, taken up by the liver, and, in part, converted to glutamate. A similar placental–liver cycle exists for serine and glycine. The physiological function of these interorgan exchanges of some amino acids and their relevance to the human placenta remain to be established.[258] A mathematical model, recently applied to amino acid transport in the human placenta and validated using experimental data, indicated that the flux of serine and alanine is particularly sensitive to changes in transporter abundance in the BM.[262]

Neutral Amino Acids

Classical studies have identified System A as a major sodium-dependent transporter that mediates the uptake of small zwitterionic nonessential neutral amino acids into the cell. System A is pH sensitive, and it displays extensive hormonal and adaptive regulation. System A activity establishes the high intracellular concentration of nonessential amino acids, which are used to exchange for extracellular essential amino acids via System L. Thus, System A activity is critical for cellular uptake of both nonessential and essential amino acids. There are three isoforms of System A: sodium-dependent neutral amino acid transporter 1 (SNAT1), SNAT2, and SNAT4 (Table 39.2). Of these isoforms, SNAT1 and SNAT2 share extensive similarities in substrate profiles and transport mechanism, although SNAT2 is more widely expressed than SNAT1. SNAT4 also transports cationic amino acids, independent of Na^+, and is predominantly expressed in the liver. However, all SNAT isoforms, including SNAT4, are expressed in the human placenta.[184] System A activity is present in both the MVM and BM; however, its activity in the BM is markedly lower.[185,186] These observations are consistent with studies using immunohistochemistry, localizing SNAT isoforms to the MVM.[183] Although SNAT1 was reported to exhibit the major System A activity in cultured primary human trophoblasts,[263] these findings are not entirely consistent with recent studies showing that silencing of mammalian target of rapamycin complex 1 and 2 (mTORC1 and mTORC2, respectively) in primary human trophoblasts completely inhibits System A activity and markedly decreases SNAT2 expression in MVM without affecting SNAT1 or SNAT4 expression,[264] implicating SNAT2 as the predominant contributor to System A activity. Additional work is needed to determine the relative contribution of each SNAT isoform to System A activity in vivo and in vitro.

System ASC transports alanine, serine, and cysteine in a Na^+-dependent manner. While ASC activity has been demonstrated in the BM,[186] ASC function in the MVM remains unclear.[185] The expression of ASCT2, which has been implicated as the primary transporter responsible for ASC activity in the human, is very low in the placenta, suggesting that an as-yet-unidentified ASC-like transporter may be responsible for the ASC activity in the placenta. System B^0 is another Na^+-dependent amino acid transporter that is highly expressed at the mRNA level in the human placenta. Although B^0 activity is clearly present in choriocarcinoma cell lines, it remains to be established if this transporter is active also in syncytiotrophoblast plasma membranes.[192] The data also remain controversial with respect to System N activity.[187,188] Na^+-dependent glycine uptake mediated by System Gly has been reported in the MVM.[189]

The β-amino acid taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in many tissues. Taurine is not incorporated into proteins and plays an important role in bile acid conjugation, defense against oxygen free radicals, regulation of neuronal excitability, and cell volume regulation. Taurine deficiency in fetal life is associated with growth failure, abnormal cellular development, retinal degeneration, cardiac damage, and central nervous system (CNS) dysfunction. The capacity to synthesize taurine is low in the human fetus, and transplacental transfer of taurine is thus the primary source of this important amino acid for the fetus. System β mediates cellular uptake of taurine, Na^+, and Cl^-, and this transporter is highly active in the MVM. In contrast, System β activity in the BM is only 6% of the MVM's activity.[190,191]

The System L amino acid transporter is a sodium-independent exchanger that mediates cellular uptake of essential amino acids, including leucine, methionine, and tryptophan. In fact, because no accumulative transporters that transport essential amino acids are expressed in the MVM, System L exchange of nonessential amino acids in the syncytiotrophoblast cytosol with extracellular essential amino acids is critical for the uptake of essential amino acids across the MVM. This transporter is a heterodimer, consisting of a catalytic light chain, typically LAT1 (large neutral amino acid transporter 1, also known as *SLC7A5*) or LAT2 (also known as *SLC7A8*), and a heavy chain, 4F2hc/CD98 (*SLC3A2*). The heavy chain is believed to be important for trafficking of the light chain to the plasma membrane, where the two subunits form disulfide-bound heterodimers. System L activity has been reported in both the MVM and BM.[185,186,192]

Kudo and Boyd reported that MVM System L activity is due to expression of LAT1,[265] whereas other investigators have suggested that LAT2 is the predominant isoform in the MVM.[266] Lastly, the efflux transporter LAT4 is expressed in the placenta, and is functional in isolated perfused human placental cotyledons, suggesting that LAT4 may also play a role in the efflux of certain essential amino acids across the BM.[193]

Cationic Amino Acids

Cellular uptake of the cationic (or basic) amino acids lysine, arginine, and histidine is driven by the electric potential difference (negative intracellularly) across the plasma membrane. System y^+ is the main transporter for cationic amino acids in the MVM,[194,195] whereas y^+L is likely to be the primary transporter in the BM.[195,196] However, System y^+L activity also has been identified in the MVM,[194,195] and System y^+ activity has been detected in the BM by some but not all investigators.[194,195] Whereas System y^+ only accepts cationic amino acids, y^+L transports neutral amino acids in the presence of Na^+. Whether or not System $b^{0,+}$ activity is present in syncytiotrophoblast membranes remains controversial.[195,196]

Anionic Amino Acids

The anionic (acidic) amino acids glutamate and aspartate are not transferred in the maternal–fetal direction in the in vitro perfused placenta. Furthermore, glutamate is rapidly oxidized by cultured primary human trophoblasts, and there appears to be no net flux of glutamate from the placenta into the umbilical circulation in pregnant women.[267] Collectively, these findings suggest that glutamate is not maternally derived, but originates from the fetal liver. Glutamate may be taken up from both the fetal and maternal circulation into the syncytiotrophoblast, where glutamate is oxidized or converted to glutamine. Consistent with this model, activity for System X^-_{AG} transporters, which mediate cellular uptake of anionic amino acids driven by the Na^+ gradient and associated with co-transport of H^+, have been shown to be present in both the MVM and BM.[171,197]

Regulation of Amino Acid Transporters

The System A transporter has been the primary focus of research into mechanisms regulating placental amino acid transport. System A transporter activity in human trophoblasts is stimulated by insulin, IGF-I, and EGF, and by substrate concentrations.[250] Leptin increases System A activity in primary villous fragments at term. The proinflammatory cytokines interleukin 6 (IL6) and TNFα also stimulate trophoblast System A activity, and the effect of IL6 was shown to be mediated by signal transducer and activator of transcription 3 (STAT3). Adiponectin decreased insulin-stimulated System A amino acid uptake in cultured human trophoblasts by modulating insulin receptor substrate phosphorylation. Furthermore, chronic administration of adiponectin to pregnant mice inhibits placental insulin and mTORC1 signaling, downregulates the activity and expression of System A and L isoforms, and decreases fetal growth.[268] Dexamethasone, a synthetic glucocorticoid, stimulates System A activity in cultured primary human trophoblasts and in term villous explants. Notably, dexamethasone administered to pregnant mice downregulates placental System A amino acid transport,[269] suggesting that the effects of glucocorticoids on System A activity in vitro and in vivo are distinct. Furthermore, in cultured primary human trophoblasts, hypoxia decreases System A activity, which could be explained by a decreased protein expression of the two System A transporter isoforms SNAT1 and SNAT2.[270] Lastly, oleic acid activates System A activity in cultured primary human trophoblasts, an effect mediated by Toll-like receptor 4.[271]

The mTOR signaling pathway responds to changes in nutrient availability and growth factor signaling to control cell growth and metabolism. Rapamycin inhibits mTOR and markedly decreases System A activity in primary human trophoblasts without affecting global protein expression of SNAT isoforms. These findings are consistent with the possibility that mTOR regulates amino acid transporter activity at the posttranslational level. Indeed, inhibition of mTOR using gene-silencing approaches decreases the activity of key placental amino acid transporters in primary human trophoblasts, an effect mediated by modulating the trafficking of specific transporter isoforms between the cell interior and the plasma membrane.[264]

The regulation of placental amino acid transporters other than System A has been insufficiently explored. Studies of the effect of insulin on System L activity in primary human trophoblasts have produced inconsistent results. However, mTOR is a powerful positive regulator of trophoblast System L activity, mediated by influencing LAT1 trafficking to the plasma membrane.[264] The nitric oxide donor SIN1 inhibits taurine transport in MVM vesicles and in villous explants. mTOR increases mRNA levels of the taurine transporter, and stimulates taurine transport in cultured trophoblasts.

Lipids

Dietary fat and fat derivatives are essential for human development,[272] as humans cannot synthesize the diverse types of fat that are essential for development and normal physiology. Fat precursors such as fatty acids are essential for energy storage, membrane phospholipids, signaling molecules (e.g., eicosanoids), and steroid receptor ligands. Sterols such as cholesterol are also crucial during early and late embryonic development.[273] Cholesterol regulates organogenesis by activation of hedgehog

signaling pathways, and is required for fetal growth, organelle membranes, steroid hormone synthesis, and production of bile salts. While the fetus is capable of cholesterol synthesis, most sterol derivatives are transferred to the embryo transplacentally. This transport process involves uptake by the trophoblast, trafficking through the syncytium, basal membrane, and endothelial cells into the fetal circulation (Figure 39.15). As noted in this chapter, there are more data on processes that occur at the level of uptake and trophoblastic transport, and less about efflux and utilization of lipids within trophoblasts.

Triglycerides, low-density lipoprotein (LDL) cholesterol, and apolipoprotein B are significantly increased in the maternal serum and tissues during early pregnancy. Accelerated fetal growth during the second half of pregnancy is associated with maternal lipid catabolism, coupled with relative insulin resistance and increased hepatic production of triglycerides. These triglycerides are available for transplacental transport to the fetus. For sufficient quantities of fatty acids to be absorbed into placental syncytiotrophoblasts, lipoproteins are hydrolyzed by triglyceride hydrolase at the MVM, which releases fatty acids from triglycerides in LDLs and very-low-density lipoproteins.[274] The nonuniform fetal-to-maternal plasma ratios for different types of fatty acids suggest dissimilar rates of transport and metabolism for different fatty acids.

FAT/CD36 (fatty acid translocase and cluster of differentiation 36) is expressed in both the MVM and BM of

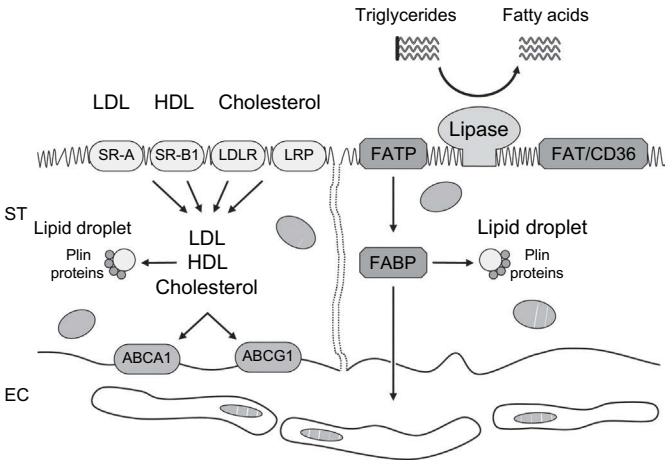

FIGURE 39.15 Lipid trafficking within placental trophoblasts. Triglycerides are cleaved by lipases at the maternal surface of the placenta. Fatty acids are taken up into cells by fatty acid transport proteins (FATPs) and FAT/CD36. These fatty acids are then carried and directed by fatty acid binding proteins (FABPs) to intracellular targets, such as lipid droplets or the nucleus, or are shuttled to the fetal circulation. Cholesterol and lipoproteins are taken up into the syncytiotrophoblast (ST) by LDL receptors (LDLRs), LDL receptor-related proteins (LRPs), scavenger receptor A (SRA), and HDL-binding scavenger receptors B1 (SRB1s). Some cholesterol is retained in the cells and stored in lipid droplets. ATP-binding cassette (ABC) transporters ABCG1 and ABCA1 mediate cholesterol efflux to fetal capillaries (ST: syncytiotrophoblast, EC: Endothelial cells).

trophoblasts,[274] where it binds lipoproteins, long-chain fatty acids, and oxidized LDLs, and facilitates their transport. The six members of the fatty acid transport proteins (FATPs) family regulate fatty acid transport, and are particularly germane at low-fatty-acid concentrations, when diffusion is insignificant. The family includes six integral transmembrane proteins that are expressed in a tissue-specific manner. FATP1–4 and FATP6 are expressed in the human placenta, with subtype-specific expression in the MVM and BM. Intracellular mobilization of fatty acids, eicosanoids, and other lipids within the hydrophilic cytoplasm is orchestrated by fatty acid binding proteins (FABPs),[275] a family of abundantly expressed small (14–15 kD) intracellular transport proteins. FABP1, FABP3, FABP4, FABP5, and FABP7 are expressed in the human placenta and in cultured human trophoblasts. The upregulation of FABP1, FABP3, and FABP4 in hypoxic trophoblasts suggests a role in the fat accumulation observed in the hypoxic placenta. FABPpm is expressed at the microvillous plasma membrane and thereby can preferentially bind and facilitate the uptake of selected fatty acids.[274]

The MVM of human trophoblasts expresses proteins that play a pivotal role in the uptake and trafficking of cholesterol. These include LDL receptors, LDL receptor-related proteins, scavenger receptors A (SRAs), and high-density-lipoprotein (HDL)-binding scavenger receptors BP (SRB1s). Cholesterol is taken up by trophoblasts via internalization of receptor-bound apoB- or apoE-carrying lipoproteins and oxidized LDL, and from SRB1-bound HDL. It is subsequently released on the BM side. The ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1, are regulators of cholesterol efflux. These proteins are also expressed in human placental endothelial cells. Inhibition of either ABCA1 or ABCG1 activity diminishes cholesterol efflux and fetal lipoprotein levels, pointing to a role in regulating the level of lipoproteins in the fetal blood.[276]

Trophoblasts also store lipids within lipid droplets. These dynamic organelles (average size 1–100 μM) are formed within the endoplasmic reticulum, and serve as an intracellular reservoir for neutral lipids, including triglycerides, cholesterol esters, and retinol esters. They also serve to protect the cells from lipotoxicity.[277] The lipid droplets are coated by a phospholipid monolayer and regulatory proteins. These proteins respond to environmental exposures and cellular needs, shuttle lipids from the endoplasmic reticulum to the lipid droplets, and control lipid droplet stability, as well as access and function of lipases.[278,279] Among these proteins, two members of the Plin family of proteins, Plin2 (adipophilin, adipose differentiation-related protein) and Plin3 (tail-interacting protein 47), are most prevalent in human trophoblasts, and likely govern the formation, size, and distribution of trophoblastic lipid droplets and lipid

accumulation. Lipolysis and release of fatty acids by the lipid droplet are accomplished by cytoplasmic lipases, such as hormone-sensitive lipase (HSL) and adipocyte triglyceride lipase (ATGL), which catabolize triacylglycerols to fatty acids and glycerol. Although the processes underlying lipolysis at trophoblastic lipid droplets are incompletely understood, expression analysis suggests that ATGL, and not HSL, is the dominant lipase in trophoblasts, where it stimulates lipid droplet lipolysis. Comparative gene identification-58 (CGI58), also known as α/β-hydrolase domain containing-5 (ABHD5), is a lipid droplet protein that is tethered to lipid droplets via its interaction with Plin proteins. Upon activation, CGI58 translocates to ATGL and activates lipolysis.[280] Whereas ATGL deletion in the mouse does not lead to a placental phenotype, deletion of CGI58 causes fetal growth restriction.[281,282]

Trophoblastic lipid transport and metabolism are under the control of endocrine signals. These include leptin, which is characteristically upregulated in the maternal plasma during pregnancy, likely reflecting leptin synthesis by the placenta. The fetus also produces leptin, and fetal leptin plasma levels correlate with birth size. Leptin is also subject to regulation by other hormones, including insulin, glucocorticoids, and thyroid hormone, suggesting that leptin is a part of the hormonal homeostatic response. Adiponectin has been shown to decrease the expression of lipoprotein lipase in cultured trophoblasts.[283] Resistin and ghrelin are expressed in trophoblasts, yet their role in placental lipid biology remains largely speculative.[284] Lastly, it is noted that several transcription factors that modulate the expression of genes involved in lipid trafficking in trophoblasts and adipose and other tissues, such as peroxisome proliferator-activated receptors and retinoid X receptor alpha, have a central role in placental development and function, supporting the notion that lipid uptake and trafficking are essential for placental function and fetal growth.[285]

Vitamins and Micronutrients

Folate

Cellular uptake of folate is mediated by the folate receptor alpha (FRα), proton-coupled folate transporter (PCFT; SLC46A1), and reduced folate carrier (RFC; SLC19A1), which have been shown to be expressed and active in the human placenta.[286] Placental FRα is predominantly localized to the MVM[286] and mediates folate uptake via receptor-mediated endocytosis. Endocytotic vesicles subsequently travel through the endocytotic pathways (early and late endosomes and lysosomes), during which the interior acidifies, causing the release of folate from FRα. Folate then exits the endosome and lysosome mediated by PCFT, driven by the large proton gradient between the endosome–lysosome and the cytosol. PCFT is highly expressed in the apical plasma membrane of the intestinal epithelium and plays a critical role in folate absorption from the intestinal lumen. In the placenta, PCFT is expressed in several cell types. In the syncytium, PCFT is polarized to the MVM.[286] PCFT cotransports folate and protons, and requires a proton gradient to efficiently transport folate. Because the proton gradient across syncytiotrophoblast plasma membranes is outwardly directed,[162] it is unlikely that PCFT plays a significant role in folate uptake in syncytiotrophoblasts. However, PCFT may be involved in transplacental folate transport by virtue of its role in folate efflux from endosomes and lysosomes. RFC is an anionic exchanger, mediating the cellular uptake of folate in exchange for anions such as organic phosphates. RFC has been proposed to be the major route for cellular folate uptake at physiologic pH. RFC is expressed in the MVM and BM,[286] and likely plays a role in folate uptake across the MVM in exchange for intracellular organic phosphates. The physiological role of RFC in the BM remains to be established. It is likely that ABC transporters mediate folate efflux across the BM. For example, multidrug resistance-associated protein 1 (MRP1), known to transport folate, is highly expressed in the BM.[287]

Vitamin B12

Vitamin B12 is bound to specific carrier proteins, in particular transcobalamin II (TCII). Placental transport has been studied both in vitro and in vivo,[288,289] and takes place via high-affinity TCII–B12 complex receptors.[290] The TCb1R receptor, which mediates cellular uptake of B12, is expressed in the human placenta.[291]

Iron

Iron uptake is critical for the feto-placental unit, as iron deficiency impairs heme synthesis, suppresses mitochondrial energy production, and inhibits cell proliferation. In contrast, excess free iron can cause oxidative damage to membranes, proteins, and lipids. Placental iron transport has been investigated in a range of species (reviewed in Refs 292,293). Diferric (Fe^{3+}) transferrin in the maternal circulation binds to the transferrin receptor in the MVM and is internalized by clathrin-mediated endocytosis. After acidification of the iron-carrying endosome, iron is reduced (Fe^{2+}) and released from the transferrin receptor, which is recycled to the plasma membrane. Efflux of iron from the endosome is believed to be mediated by the divalent metal transporter protein (DMT1, aka SLC11A2). However, other transport mechanisms are likely to play a role in iron trafficking, as mice with homozygous deletion of DMT1 are born with similar iron stores as wild-type controls. Once in the cytoplasm, the iron may be used in biosynthetic pathways or stored (bound to ferritin or as free iron). Alternatively, iron is oxidized by endogenous ferroxidase and transported by ferroportin, also known

as iron-regulated gene (IREG1) or metal transport protein (MTP1),[292,294] across the BM to the fetus. During periods of iron deficiency, the fetus has the capacity to upregulate the transferrin receptor in the MVM and DMT1 in endosomes to ensure adequate transport to the fetus.

Drugs and Xenobiotics

Like many other organs, placental trophoblasts express transporters that regulate placental uptake as well as efflux of drugs and xenobiotics. Unlike other organs, the placenta has the unique role of governing the transport of these drugs into the fetal compartment, and protecting it from adverse effects. In addition to transporter expression, the transfer process depends upon the physico-chemical properties of the drug, placental blood flow, and drug metabolism locally within trophoblasts, or within the maternal or fetal compartments.[295–297] The precise mechanism of action of most placental drug transporters remains unclear. As discussed elsewhere in this chapter, ethical considerations prohibit direct drug transport studies in humans in vivo, and inferences are made from studies in other systems, including ex vivo placental perfusion, cultures of primary human trophoblasts from early or late pregnancy, trophoblast cell lines, trophoblast membrane vesicles, and animal models in vivo (reviewed in Refs 298,299).

Many drug transporters are expressed within the MVM, BM, or endothelial cells within the placenta. These are members of the phospho-glycoprotein family (P-gp: multidrug resistance proteins MDR1 and ABCB1), multidrug resistance–associated proteins (MRP proteins), breast cancer resistance protein (BCRP, aka ABCG2), and other drug and xenobiotics transporters, including organic anion-transporting polypeptides, organic anion transporters, organic cation transporters, monocarboxylate transporters, equilibrative nucleoside transporters, and folate transporters (reviewed in Refs 298,300). Two ABC proteins, P-gp and BCRP, are the best characterized efflux transporters in the placenta and play a critical role within trophoblasts (reviewed in Refs 300–302). P-gp and BCRP are membrane-spanning transporters, which are expressed primarily in the MVM of the syncytiotrophoblast, where P-gp binds a range of organic cations, carbohydrates, chemotherapeutics, antibiotics, and steroids. BCRP overlaps with P-gp in binding a number of endogenous and exogenous drugs and xenobiotics.[303,304] Although the mechanism of action of these two transporters remains unclear, they seem to prevent the entry of drugs into the placenta by effluxing them back into the maternal circulation. These proteins also play a role in transplacental transport of relevant drugs, such as synthetic steroids and glyburide.[301] The pathways that regulate the expression of these transporters remain unclear. While these transporters are known to be modulated by diverse transcription factors, steroid hormones and pathological conditions such as placental infection, hypoxia, and possibly epigenetic mechanisms, the pathways regulating their expression are undetermined. Interestingly, the expression of P-gp declines before term.[301] Lastly, MRPs represent a subfamily of ABC transporters, encoded by genes ABCC1–13. MRPs transport a number of organic anions, and have some overlapping substrates with P-gp and BCRP. Members of this family of transporters are expressed in the MVM, BM, and placental endothelial cells.

The aryl hydrocarbon receptor is a nuclear hormone receptor that heterodimerizes with the ARNT to regulate gene expression in response to diverse drugs. These proteins are expressed in trophoblasts, where they bind many environmental endocrine disruptors that are implicated in placental injury and fetal growth disorders, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and diverse types of cigarette smoke hydrocarbons. Targets for these transcription factors include key placental genes that are involved in drug metabolism, such as cytochrome P450 enzymes CYP1A1, CYP1A2, and CYP1B1; glutathione S-transferase (GST1); UDP–glucuronosyl transferases; and others.[305–307]

The serotonin transporter (SERT) and the norepinephrine transporter (NET) are monoamine transporters attracting more research in recent years, as their role in modulating the action of cocaine and antipsychiatric drugs became apparent. Cocaine, amphetamines, and their derivatives cause increased sympathetic activity, which results in vasoconstriction and decreased uterine blood flow, as well as stimulation of uterine contractile activity. Indeed, both fetal growth restriction and preterm delivery are complications of abuse of cocaine and its derivatives.[308] Both SERT and NET are expressed in the MVM.[309] In the brain, serotoninergic and noradrenergic transporters on presynaptic cells clear the synaptic space from the monoamines, preventing excessive neurotransmitter activity. It is thus assumed that the syncytiotrophoblastic transporters have a similar function, and clearance of serotonin and norepinephrine by the "placental sink" leads to entry of these chemicals into the syncytiotrophoblast, where they can be cleared by the abundant monoamine oxidases.[310] A lack of clearance induced by transporter inhibitors (amphetamines or cocaine) may therefore lead to excess vasoactive substances in the intervillous space, and their adverse consequences.[308]

PLACENTAL TRANSPORT IN COMPLICATIONS OF PREGNANCY

Disorders Associated with Placental Dysfunction

A number of different clinical conditions are associated with placental maldevelopment and dysfunction.

While these conditions have distinctive clinical presentations, their placental histopathological manifestations overlap. Thus, in most cases, placental histological lesions may not be specific enough to define the specific disease associated with placental dysfunction. The most common disorders reflecting placental dysfunction are fetal growth restriction, intrauterine fetal death, and preeclampsia (discussed here). Evidence for placental hypoperfusion characteristic of these clinical conditions, suggesting that they are part of a spectrum of placental pathologies linked to early impairment of spiral artery conversion or late impairment of villous perfusion and trophoblast function.[89,311–313] Hypoperfusion lesions typically appear as clustered apoptotic trophoblasts ("syncytial knots"), disordered villous maturation with subsequent ischemic villous agglutination, and possibly increased intervillous fibrin. These lesions progress with worsened hypoxia, causing further fibrin deposition and atherosis of the decidual arteries. Profound and early impairment of trophoblast invasion and inadequate spiral arterial dilation are associated with spontaneous miscarriages or stillbirths due to premature and disorganized onset of the maternal circulation, whereas less severe deficiencies are associated with intrauterine growth restriction and preeclampsia.[89,314,315] In addition, suboptimal fetal growth, fetal injury, and stillbirths may also reflect abnormalities of the fetal vasculature within the placenta, with fetal vessel thrombi, villous inflammation, or avascular villi.[316,317]

Oxidative stress likely plays a role in the pathophysiology of pregnancy disorders associated with placental dysfunction.[311,318–321] Physiological measurements performed in vivo during the first trimester of human pregnancy indicate that the trophoblast is well adapted to a low-oxygen environment.[64,322] Indeed, it appears that fluctuating oxygen concentrations within the intervillous space caused by intermittent perfusion from the spiral arteries may cause hypoxia–reoxygenation and villous injury.[89,323–325] Oxidative stress with relative hyperoxia or hypoxia is associated with reduced cytotrophoblast proliferation and increased rate of apoptosis. As the trophoblast includes a regenerative layer of cytotrophoblasts, the ultimate consequences of oxidative stress depend on the balance of syncytial degeneration and regeneration.[325]

Among pregnancy disorders that are associated with placental dysfunction, preeclampsia stands out because of its distinctive effect on the mother. Preeclampsia is a pregnancy-specific disease that manifests as hypertension, excessive edema, and proteinuria. When the disease worsens, it causes target organ damage, commonly affecting the kidneys, liver, blood cells and coagulation, cardiopulmonary system, and CNS, where it may manifest as seizures (eclampsia). Preeclampsia is also associated with fetal growth restriction and its sequelae. Preeclampsia is attributed to reduced invasion of extravillous trophoblasts and abnormal vascular remodeling early in pregnancy.[311,319,320] The appearance of preeclampsia only in pregnancy, its abatement shortly after delivery, and the association of preeclampsia with large placental mass and, uniquely, with molar pregnancy, all support the notion that the placenta harbors the cause of preeclampsia. Given the association of preeclampsia with maternal vascular disease, it is speculated that reduced placental perfusion may cause placental injury, and thereby preeclampsia, or predispose the mother to preexisting adverse signals from the placenta.[326]

The reasons for the unique maternal aspects of preeclampsia, despite a placental histopathology that is commonly shared with fetal growth restriction, are puzzling.[327,328] In addition, the vascular-remodeling theory of preeclampsia remains uncertain, as reduced dilation of the distal part of the spiral arteries may not have a major effect on blood flow.[329,330] Conflicting data also exist on secreted products of placental oxidative stress, such as sFlt or PlGF, and their causal role in preeclampsia.[331–333] Interestingly, preeclampsia is characterized by increased release of syncytiotrophoblast microparticles, which are implicated in maternal endothelial cell injury.[319,334–336]

Whereas preterm birth is the most important cause of morbidity and mortality of viable fetuses,[337] the role of the placenta in this disease remains unclear. Among all etiologies of preterm birth, intraamniotic infection and a secondary inflammatory response are established causes of preterm birth. This may manifest as placental inflammation, mainly in the chorionic plate, fetal membranes, and umbilical cord. The association of inflammation with amniotic necrosis, villous inflammation, and thrombosis further adds to the fetal insult.[338,339] Decidual hemorrhage is also common in spontaneous preterm birth.[340] Placental injury associated with fetal growth restriction, preeclampsia, and evidence for poor fetal oxygenation are typically associated with induced, medically indicated preterm birth. The following discussion centers on clinical conditions associated with altered placental nutrient transport, which may contribute to fetal growth abnormalities or fetal injury.

Placental transport functions have been studied extensively in fetal growth restriction associated with placental injury.[172,173] In many of these studies, investigators have determined the activity of transporters in isolated MVM and BM, using plasma membrane protein concentration as the denominator, thereby comparing the transport capacity per unit membrane area of healthy and growth-restricted fetuses (Table 39.3). Thus, changes in the effective placental exchange area are not adequately considered in such studies. Some growth-restricted fetuses are hypoglycemic in utero[159]; however, this appears not to be due to a decreased glucose transport capacity. In contrast, fetal growth restriction due to maternal hypoxemia at high altitude has been associated

TABLE 39.3 Alterations in Placental Transport in Human Fetal Growth Restriction

Transport System	MVM	BM
Glucose	↔	↔
System A	↓	↔
System L	↓	↓
System β	↓	↔
Lysine	↔	↓
Lipoprotein lipase	↓	nd
Ca^{2+} ATPase	nd	↑
Na^+/H^+ exchanger	↓	nd
Lactate	↔	↓
Na^+/K^+ ATPase	↓	↔

Increased (↑), unaltered (↔), or reduced (↓) transporter activity per milligram membrane protein in microvillous (MVM) and basal plasma membranes (BM) isolated from human pregnancies complicated by fetal growth restriction at term as compared to appropriate-for-gestational age (AGA) controls. nd: not determined.
Reviewed in Refs 172,173.

with decreased expression of glucose transporters in the BM, indicating decreased glucose transport capacity.[341]

System A activity has been shown consistently to be lower in MVM isolated from placentas of growth-restricted fetuses (Table 39.3). Importantly, the most severe cases of fetal growth restriction were associated with pronounced decreases in MVM System A activity. In contrast, System A activity is not altered in villous explants obtained from placentas of growth-restricted fetuses in pregnancies complicated by preeclampsia.[342] Preeclampsia, but not fetal growth restriction without preeclampsia, is typically associated with increased maternal levels of insulin and leptin, which are known to stimulate placental System A activity in vitro.[343,344] Thus, it is possible that the distinct hormonal profile of women with preeclampsia may support placental transport functions in this disease. Homocysteine is a competitive inhibitor of System A transport,[345] and uric acid has been reported to decrease System A activity in villous fragments mediated by intracellular redox-signaling pathways.[346] Thus, elevated maternal serum levels of homocysteine and uric acid, which are often observed in this pregnancy complication, may decrease placental System A activity in vivo.

The activity of transporters of essential amino acids, including System β (transporting taurine) and System L (transporting a range of essential amino acids, including leucine and phenylalanine), is reduced in MVM and/or BM isolated from placentas associated with fetal growth restriction (Table 39.3). These in vitro findings are supported by studies in pregnant women using stable isotopes, demonstrating that placental transfer of the essential amino acids leucine and phenylalanine is reduced in fetal growth restriction at term.[347] In addition, reduced placental capacity to transport amino acids is consistent with studies showing reduced concentrations of amino acids, in particular essential amino acids, in the umbilical vein of growth-restricted fetuses.[267] The activity of MVM lipoprotein lipase, which mediates the first critical step in transplacental transfer of free fatty acids, is also reduced in fetal growth restriction. These findings are in line with clinical studies showing lower fetal–maternal plasma ratios for long-chain polyunsaturated fatty acids in fetal growth restriction.[348]

Placental ion transporters are also subjected to regulation in fetal growth restriction. For example, the activities of Na^+–K^+–ATPase, the Na^+–H^+ exchanger, and lactate transporters are decreased in syncytiotrophoblast plasma membranes isolated from placentas of growth-restricted fetuses. The reduced Na^+–K^+–ATPase activity may increase intracellular concentrations of Na^+, thereby adversely affecting all transport processes dependent on the Na^+ and electrical gradient. Growth-restricted fetuses are prone to develop acidosis in utero, which may be due, in part, to the decreased activity of placental transporters that are critical for the elimination of protons from the fetal circulation. Other ion transporters, however, appear to be regulated quite differently. BM Ca^{2+}–ATPase is upregulated in placentas associated with fetal growth restriction, which may be caused by increased fetal concentrations of PTHrP (38–94),[232] a key regulator of the placental calcium pump.

Obesity

Obesity commonly affects pregnant women worldwide and increases the risk of larger (macrosomic) fetuses, along with related structural and metabolic abnormalities. The mechanisms linking excess maternal adiposity and fetal overgrowth are poorly understood, and the effect of maternal overweight and obesity on placental function remains largely unknown.[349] System A, but not System L, amino acid transport activity is increased in MVM isolated from placentas of obese Swedish women giving birth to large babies.[350] This finding correlated with the activity of placental insulin–IGF-I and mTOR signaling, whereas AMP-activated protein kinase phosphorylation was inversely correlated to birth weight.[350] Using villous fragments, other investigators have reported decreased placental System A activity in overweight or obese Hispanic women giving birth to normal-sized babies.[351] Studying placental transport in the placentas of obese mothers giving birth to normal-sized babies, Dube and coworkers found that placental lipoprotein lipase (LPL) activity and CD36 expression increased, yet placental expression of FATP4, FABP1, and FABP3 decreased.[352]

Additional studies are needed to allow firm conclusions with respect to the impact of maternal obesity on placental nutrient transport.

Diabetes

With the exception of women with type 1 diabetes who develop vascular complications, diabetes in pregnancy, in particular gestational diabetes, is associated with fetal overgrowth. Most studies suggest an increase in placental capacity to transfer nutrients in diabetes associated with fetal overgrowth; however, the data with respect to amino acid transport are less consistent (Table 39.4).

System A amino acid transport activity is reduced and System L transport activity is unaltered in MVM isolated from pregnancies with type 1 diabetes and fetal overgrowth.[172] In contrast, other investigators reported increased MVM System A transporter activity in Swedish women with type 1 diabetes, independent of fetal overgrowth. In addition, placental transport of leucine is increased in women with gestational diabetes. These inconsistent patterns may be related to methodological differences or to variations in study populations. Glucose transport activity and GLUT1 expression in the BM are increased in type 1 diabetes. Because the transport across the BM is likely the rate-limiting step in placental glucose transport, these changes are predicted to increase glucose delivery to the fetus, and they may contribute to fetal overgrowth in type 1 diabetes. Furthermore, the expression of GLUT9 is increased in the MVM and BM isolated from the placentas of women with diabetes,[181] supporting the evidence for increased placental glucose transport capacity associated with this pregnancy complication.

As discussed in this chapter, maternal lipoproteins are the predominant source of fetal fatty acids, likely contributed by the two key membrane-bound lipid hydrolases, lipoprotein lipase, and endothelial lipase.[247] The activity of placental LPL is increased in type 1 diabetes associated with fetal overgrowth. The expression of endothelial lipase is increased in the same conditions.[353,354] In addition, FABP1 expression is upregulated in the placenta in gestational diabetes or preexisting diabetes associated with a large newborn. Similarly, placental expression of FABP4 is elevated in pregnancies of obese women with diabetes.[355] Collectively, these findings suggest an increase in placental supply of lipids to the fetus in pregnant women with diabetes. Considering the complexity of placental nutrient transport as well as the diversity of diabetes phenotypes, additional work in the field is greatly needed.

TABLE 39.4 Alterations in Placental Transport in Maternal Diabetes Associated with Fetal Overgrowth

Transport System	MVM	BM
System A	↑, ↓	↔
System L	↑,[a] ↔	↔
System β	↔	↔
Lysine	↔	↔
Glucose	↔	↑[b]
Lipoprotein lipase	↑	nd
Ca^{2+} ATPase	nd	↑
Na^{+}/K^{+} ATPase	↔	↔

Transporter activity per milligram of membrane protein was measured in isolated microvillous plasma membrane (MVM) and basal plasma membrane (BM) vesicles. The table shows the transport activity in cases of fetal overgrowth in relation to appropriate-for-gestational age (AGA) controls: increased (↑), unaltered (↔), or reduced (↓) transporter activity, nd: not determined.
[a]*Only GDM.*
[b]*Only type 1 diabetes.*
Reviewed in Refs 172,173.

CONCLUSION

Although the placenta forms the critical interface between the fetus and the mother, and is thus essential for the development, survival, and growth of all eutherian embryos, our understanding of placental development and function remains rudimentary. As a rapidly evolving organ, marked differences in placental morphology and function among eutherian embryos may challenge researchers seeking a useful animal model to study the human placenta. Conversely, deciphering evolutionary differences in placental function, when correlated with fetal development and postnatal adaptation, may illuminate important mechanisms underlying the placental support of intrauterine development and the balance between fetal demands and limiting maternal resources.

There are two fundamentally distinct, but not mutually exclusive, models for the regulation of placental transport function in response to developmental, environmental, or pathological influences. The *fetal demand model*, which is supported by several mouse studies, postulates that placental function is primarily controlled by fetal demand.[356,357] In response to maternal undernutrition or restricted utero-placental blood flow, the limited fetal nutrient availability signals to the placenta to bolster placental nutrient transport. This model represents a classical homeostatic mechanism by which the fetus compensates for changes in nutrient availability by regulating nutrient supply (i.e., placental transport) in the opposite direction, thereby correcting for deficiencies. The *maternal supply model*[358,359] suggests that the placenta responds to maternal nutritional cues, resulting in downregulation of placental nutrient transporters in

response to maternal undernutrition or restricted uteroplacental blood flow. Fetal nutrient availability will consequently decrease, resulting in fetal growth restriction. Maternal supply therefore represents a mechanism by which fetal growth is matched to the ability of the maternal supply line to allocate resources to the fetus. Maternal signals conveying nutritional information to the placenta may include metabolic hormones such as insulin, cortisol, leptin, and adiponectin, which are known to be influenced by maternal nutrition and regulate placental transport. Other possible signals to the placenta are oxygen and nutrient levels. Placental mTOR signaling may play a role in integrating these signals and in regulating fetal nutrient availability by modulating placental growth and nutrient transport.[359] In this context, it is noted that key placental transporters for amino acids, lipids, and ions are downregulated in human fetal growth restriction (Table 39.3), which is inconsistent with a homeostatic or fetal demand model for regulation of placental transport. However, most of these studies were performed at term, or in a few cases using tissue obtained from preterm deliveries in the third trimester. It is possible that compensatory changes consistent with fetal demand signals are present earlier in pregnancy, as has been shown in mouse models of growth restriction. Moreover, the distinct upregulation of BM Ca^{2+}–ATPase activity in placentas associated with fetal growth restriction may represent a compensatory activation of the placental calcium transport system in response to increased fetal demand. Published data indicate that placental capacity to transport free fatty acids, and possibly glucose, is increased in diabetic women, particularly in cases of fetal overgrowth. Thus, information gleaned from human diseases such as fetal growth restriction or fetal overgrowth generally supports the *maternal supply model* for regulation of placental transport.

The *maternal supply* and *fetal demand* models likely coexist. Recently, Gaccioli and coworkers proposed that the placenta integrates a multitude of maternal and fetal nutritional cues with information from intrinsic nutrient-sensing signaling pathways to balance fetal demand with the ability of the mother to support the pregnancy by regulating maternal physiology, placental growth, and nutrient transport (Figure 39.16).[360] It is speculated that the evolutionary pressure for the balance between maternal and fetal needs was mainly impacted by maternal undernutrition, which was the common situation throughout human history.[360] Although these regulatory loops may also function in the opposite direction in response to maternal overnutrition, it is possible that the placental responses may not be as readily apparent in maternal obesity or diabetes as they are in maternal undernutrition. Clearly, the relative importance of maternal supply and fetal demand signals for

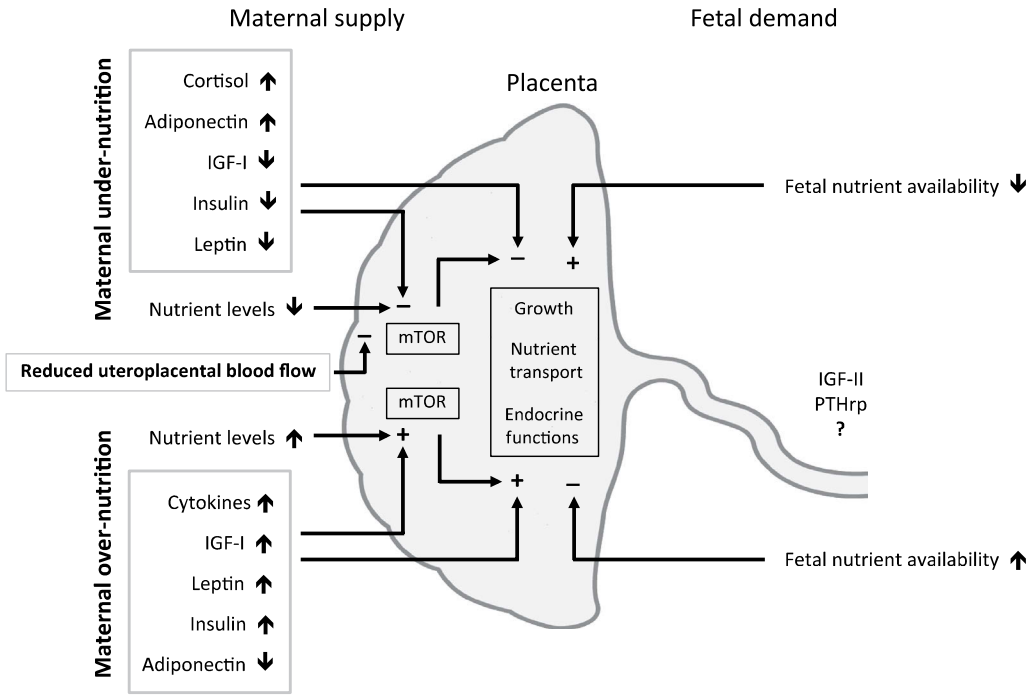

FIGURE 39.16 Maternal supply and fetal demand: an integrated model. The placenta integrates maternal and fetal nutritional signals through intrinsic nutrient sensors, such as mammalian target of rapamycin (mTOR) signaling. These signals then regulate placental growth and nutrient transport to balance fetal demand with the ability of the mother to support pregnancy. Thus, the placenta plays a critical role in modulating maternal–fetal resource allocation, thereby affecting fetal growth and the long-term health of the offspring. See text for detailed explanation. IGF: insulin-like growth factor; PTHrp: parathyroid hormone–related peptide. *Source: Reproduced with permission from Ref. 360.*

the regulation of placental function may differ between species, and depend on the type, duration, and severity of the nutritional perturbation.[360] It is plausible, for instance, that regulation by fetal demand signals is predominant when the nutritional challenge is moderate or brief, whereas regulation by maternal supply may override fetal demand signals if the nutritional challenge is more severe or prolonged. Matching fetal growth to maternal resources in situations of significant maternal undernutrition will produce an offspring that is smaller in size but which, in most instances, will survive and reproduce. Thus, rather than nutrient extraction from the already deprived mother, which will jeopardize the survival of both the mother and her fetus, restricted fetal growth might be the leading strategy.

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CHAPTER

40

Placental Endocrine Function and Hormone Action

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INTRODUCTION

The placenta differs from virtually all other organs in that it is uniquely formed by the interaction of both maternal and fetal tissues, exhibits an extreme amount of diversity among species, and coordinates and participates in a vast number of functions (e.g., metabolic, endocrine, vascular, and immunologic) that are critical to maternal well-being and fetal development. Our current understanding of the structure, development, and physiologic and endocrine roles of the placenta reflects many years of work by pioneering investigators who challenged existing dogmas using a complement of in vivo and in vitro experimental approaches and technologic methods to test hypotheses.

Notable milestones regarding the structure of the placenta include the studies of (1) William and John Hunter, who independently showed (1774–1794) that the maternal and fetal circulations in the placenta are separate and not continuous and that the "convoluted" (i.e., spiral) arteries of the uterus deliver maternal blood to the placenta; (2) William Carpenter, whose experiments delineated the intervillous space; (3) Ambrosius Hubrecht, who in 1889 defined and named the tissue barrier between maternal and fetal circulations as the trophoblast (nutrition + germ) because of its direct nutritive significance and immediate contact with maternal tissue, blood, or secreted material; (4) Carl Friedlander, who in 1870 described the presence of intravascular cells within the lumen of spiral arteries and commented that "arteries" were only rarely observed, that intravascular cells were multinuclear and might come from the placenta, and that these cells slow down or interrupt maternal blood supply to the placenta; (5) Marcus Duval, who in 1890 confirmed trophoblast invasion into maternal arteries; and (6) William Turner, who in 1876 postulated that the maternal surface of the placenta was a secreting organ that provided nutrients to the fetus and proposed that the widely accepted doctrine that substances including oxygen simply diffuse through the walls of vessels from maternal to fetal blood should no longer be accepted.[1–4]

In the early to mid-1900s, our understanding of placental structure and development, as well as the similarities and important differences between species, was brought forth by the extensive work of Winfield Mossman, Emmanuel Amoroso, Gordon Bourne, James Boyd, William Hamilton, and Allen Enders.[3,4] Concomitant studies by Louis Flexner demonstrated placental transfer of radioactive sodium to the fetus, while Elizabeth Ramsey in collaboration with Martin Donner developed and applied cineradioangiographic techniques in vivo in the rhesus monkey to visualize the pattern of maternal blood flow in the intervillous space. Ramsey's pioneering work demonstrated that unlike the labyrinth placenta of rodents in which the maternal and fetal circulations run in opposite directions and gas exchange occurs via countercurrent mechanisms, in primates, maternal arteries do not carry oxygenated blood all the way to the fetal surface of the organ. Rather, oxygenated blood is delivered to the intervillous space in spurts, or as originally described by Ramsey the "winking and

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blinking" circulation, indicating that placental perfusion is uneven,[1] a concept subsequently confirmed by several investigators.

As our understanding of the fine structure and significant vascular and transport functions of the placenta continued to increase, the concept that the placenta might also exhibit endocrine functions was initially suggested by Joseph Halban in 1905, a concept that gained significant support by the discovery of gonadotrophic activity in serum and urine of pregnant women by Ascheim and Zondek in 1927. As studies rapidly progressed to isolate and identify placental-derived protein and steroid hormones, Egon Diczfalusy and Pentti Siiteri using in vivo experimental approaches in pregnant women established in the 1960s the critical concept of a functional maternal–fetal–placental unit that highlighted the interdependence and interactions between the endocrine functions of maternal and fetal endocrine glands (EG) and the placenta. Accordingly, studies of the roles of placental hormones in key aspects of placental development, blood flow, maternal–fetal nutrient exchange, as well as timely maturation and growth of the fetus began to emerge.[3,4]

Today we recognize that the human and nonhuman primate placenta is the site of synthesis of steroid hormones and a diverse complement of peptide and protein hormones that function independently, as well as integral components of paracrine, autocrine, and endocrine control systems. During the past few decades, immunocytochemical, transgenic, and molecular advances have been employed to localize origin, define physiologic roles, and unravel the mechanisms underpinning the action of these hormones within the placenta.

This chapter will summarize the results of these recent studies; the developmental pattern and sites of expression, action, and regulation of the placental hormonal systems; and how they are integrated and function to (1) coordinate pregnancy recognition and maintenance; (2) control development of the placenta, including villous angiogenesis and remodeling of the maternal uterine spiral arteries by extravillous cytotrophoblasts; (3) regulate placental, maternal, and fetal organ systems controlling substrate availability and maternal and utero–fetal–placental vascular function; as well as (4) control the metabolism of hormones (e.g., cortisol–cortisone), thereby coordinating timely maturation of the fetal organ systems important for physiologic function in adulthood. The chapter will also summarize epidemiologic and genetic reports in humans and in vivo studies in nonhuman primate models of human pregnancy that are elucidating regulation, developmental roles, and underlying mechanisms of action of the placental endocrine system and that in humans are often associated with complications of pregnancy, for example preeclampsia, fetal growth restriction (FGR), and increased risk for diseases in adulthood.

PROTEIN HORMONES OF THE PLACENTA

The placenta produces significant quantities of the protein hormones, human chorionic gonadotropin (hCG) and placental lactogen (PL), which exhibit unique patterns of synthesis and regulate physiologic processes that are important to pregnancy. The placenta also synthesizes growth factors, the most physiologically relevant of which are insulin-like growth factor (IGF)-I and -II, transforming growth factor beta (TGFβ), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and EG-VEGF. The structures and mechanisms underlying the regulation of the synthesis and actions of hCG and PL have been presented by Ogren and Talamantes[5] in an earlier edition of the *Physiology of Reproduction*. In the present chapter, we provide an overview of the regulation of hCG and PL synthesis and recent developments on the expression and physiological actions of these hormones and the placental growth factors (PlGFs) during pregnancy.

Human Chorionic Gonadotropin

Structure

hCG is a glycosylated protein heterodimer composed of noncovalently bound α and β subunits. Syncytiotrophoblast hCG is composed of a 2-oligosaccharide 92-amino-acid α subunit and a 6-oligosaccharide 145-amino-acid β subunit, exhibits a molecular weight of 37,180, and has a half-life in circulation of approximately 36 h. The highly glycosylated α and β subunits both contain several disulfide linkages that form cysteine knot motifs, which dictate folding of the protein essential for biologic activity and thus binding to the luteinizing hormone–chorionic gonadotropin (LH–CG) receptor, as well as hormone secretion. For example, deletion of any one of the bonds in the β subunit joining cysteines 9 and 90 (9–90), 34–88, or 38–57 decreases, whereas deletion of cysteine 26–110 or replacement of cysteine with other amino acids increases, hormone secretion. The three-dimensional structure of hCG has been determined, and the crystal structure of the deglycosylated protein[6] is depicted in Figure 40.1.

α-HUMAN CHORIONIC GONADOTROPIN

The mature 92-amino-acid α subunit of hCG has a molecular size of 14,500 kDa, of which 17% is contributed by carbohydrate.[7] Disulfide bonds of αhCG join cysteines 7–31, 10–32, 28–60, 59–87, and 82–84.[8] The α subunit also contains two N-linked oligosaccharide chains at asn-52 and asn-78. An α subunit that does not combine with β-hCG (termed a free α subunit) is also expressed by the placenta and detected in maternal serum.[9] The increased

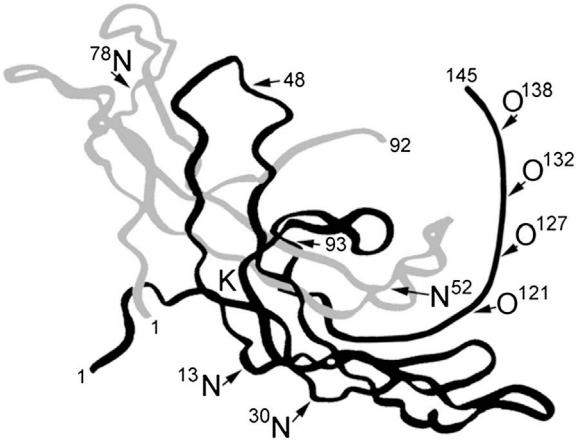

FIGURE 40.1 Crystal structure of deglycosylated regular hCG, as originally determined by Lapthorn et al.[6] and adapted by Cole.[7] The unfolded β-subunit C-terminal peptide is added (missing in crystal structure). It is inferred that this structure is not folded since the sequence comprises primarily a polymer of proline and serine residues. The symbol N indicates the attachment site of N-linked oligosaccharides, and the symbol O the attachment site of O-linked oligosaccharides. Residue β48 is indicated, the site of nicking of hCG (β47–48), and residue β93, the site of cleavage of the β-subunit C-terminal peptide (β92–93). The symbol K indicates the site of the cystine knot structure, four peptides (β1–15, β30–45, β80–100, and β50–65) linked by three disulfide bridges, β34–88, β9–57, and β38–90. The α-subunit is shown in gray, and the β-subunit is shown in black. *Source: Reprinted with permission from Ref. 7.*

size of the N-linked oligosaccharides of free α subunit is thought to be the major factor preventing dimerization with β-hCG.

The α subunit gene is located on chromosome 6q14-q21 and is expressed in the pituitary and placenta.[10] A number of regulatory elements in the 5′ flanking region are important for placental expression and regulation.[11] These include two cyclic adenosine monophosphate (cAMP) response elements (CREs)[12]; a trophoblast-specific upstream regulatory element (URE)[13]; an alpha-activator element (α-ACT)[14]; a junctional regulatory element (JRE)[15]; a CCAAT region[16]; three glucocorticoid response elements (GREs), of which only GRE2 confers glucocorticoid responsiveness[17]; and an element that spans nucleotides (nt) 244 and 213. The CREs bind nuclear proteins, including CREB-327, which is activated after phosphorylation by cAMP–protein kinase A (PKA). The URE also interacts with the CRE, but the mechanisms are not fully known. Although α subunit is also expressed in pituitary cells, expression is regulated by elements that differ from those in the placenta and include a gonadotrope-specific element (GSE).[18]

β HUMAN CHORIONIC GONADOTROPIN

The mature β subunit of hCG is composed of 145 amino acids and is structurally similar to the β subunit of pituitary LH, differing only in the terminal 28 amino acids. Six disulfide bonds of βhCG join cysteines 9 and 90 (9–90), 23–72, 26–110, 34–88, 38–57, and 93–100. The β subunit exhibits a molecular size of 22,200 kDa and contains six oligosaccharide chains, two of which are N-linked to asn-13 and asn-30 and four O-linked to ser-121, ser-127, ser-132, and ser-138. As a consequence, βhCG exhibits extensive structural heterogeneity.

The β subunit of hCG is encoded by a cluster of nonallelic genes (βhCG1, 2, 3, 5, 7, and 8), which includes the LHβ gene located on chromosome 19q13.32 (Figure 40.2(A) and (B)).[11,19–21] The βhCG genes are grouped into type 1 (βhCG7) and type 2 (βhCG3, 5, and 8) and share 97–99% DNA sequence identity.[22] The type 2 βhCG genes encode for the same protein, whereas the type 1 gene encodes a protein that differs by three amino acids, notably replacement of a proline in type 2 by a methionine. Although the βhCG1 and βhCG 2 genes share 85% identity with the other genes in the cluster, they encode a protein of 132 amino acids that exhibits no homology with the β subunit of hCG and that does not correspond to any known protein in the GenBank database.[22] Although all four βhCG type 1 and 2 genes are expressed in placental villous and extravillous trophoblasts (EVTs), βhCG5 and βhCG8 are the most actively transcribed.[19,22] Thus, 60–85% of the total βhCG mRNA extracted from placenta is βhCG5 and βhCG8 (Figure 40.2(A)).[20–22] Studies of the βhCG5 gene indicate that regulatory promoter sequences are located between nt 78 and the transcription start site,[23] with CREs at nt 311-187, nt 44-357, and nt 3700-775.[24] Suppressor activity is also present in the region at nt 287-191,[24] but the regulatory mechanisms and elements involved have not been defined. In addition to synthesis by the placental trophoblast, hCG is also expressed in other tissues, particularly the secretory phase endometrium where the βCG6 and βCG7 subunits are formed.[25] As described by Ogren and Talamantes,[5] structure–function relationships in hCG have focused on assessing subunit contact sites in the αβ dimer, receptor binding, and the role of the disulfide bonds and oligosaccharide chains.

Synthesis and Secretion

hCG is produced by the villous cytotrophoblasts prior to week 6 of gestation and primarily by the syncytiotrophoblast thereafter. Ultrastructural studies indicate that hCG is localized in secretory granules of trophoblast cells and that virtually all of the hormone after it is synthesized is released and not stored. Levels of intact hCG in maternal blood are detectable approximately 1–3 days after implantation, rapidly rise thereafter with peak levels being achieved between weeks 8 and 12 of gestation, and subsequently decline and remain relatively constant after weeks 18–20 (Figure 40.3).[26] hCG is also detected in fetal blood and amniotic fluid, and levels in both compartments show a pattern similar to that in

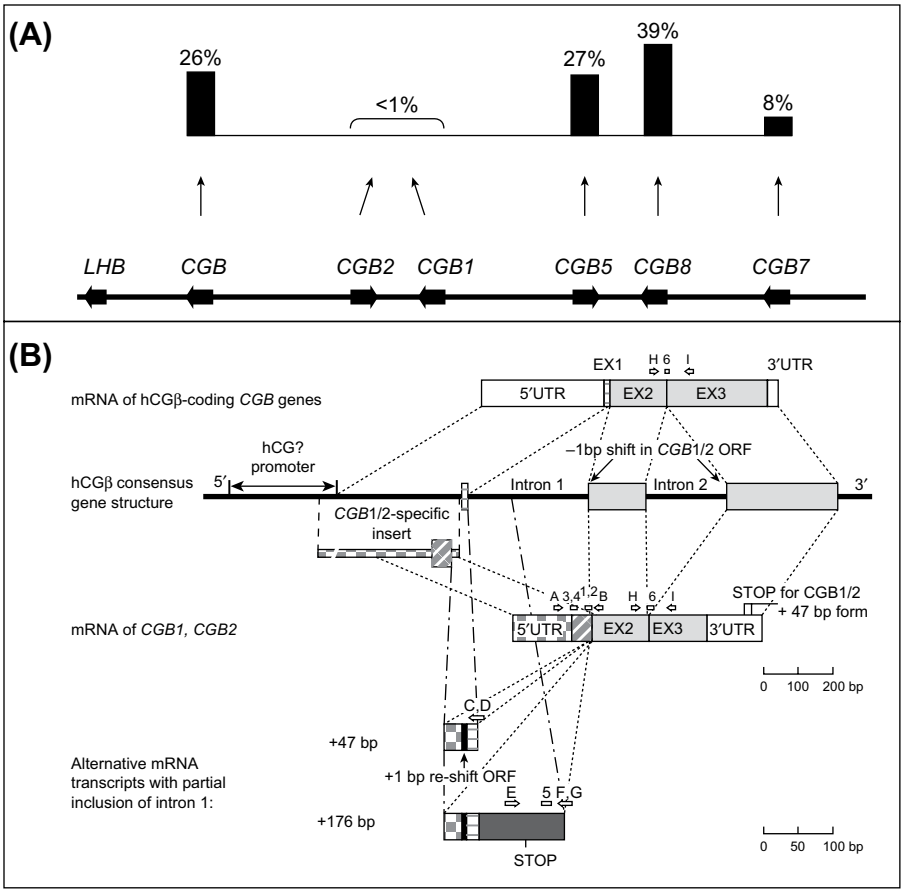

FIGURE 40.2 (A) Schematic presentation of the βLH–βhCG (*LHB–CGB*) gene cluster with genes marked as black wide arrows in the direction of the transcription on sense strand and contribution of each individual βhCG gene to the total mRNA pool. (B) Schematic representation of the structure of the βhCG genes, with coding segments marked on the consensus gene structure with gray boxes, except for the exon 1 for βhCG-coding genes (horizontal stripes) and for *CGB1–CGB2* (diagonal stripes). The 5′UTR of mRNA transcribed from βhCG-coding genes (white box) differs from the 5′UTR for *CGB1–CGB2* (checkered box); the 3′UTR of the two groups is of variable length (white box). Alternative +47 bp *CGB1–CGB2* mRNA forms contain an additional sequence from *CGB1–CGB2*-specific intron 1 (22 bp, black box), including the fragment corresponding to the hCGβ 5′UTR (10 bp, checkered box) and exon 1 (15 bp, horizontal stripes), resulting in a reshift of the *CGB1–CGB2* open reading frame (ORF) to the ORF of hCGβ-coding transcripts. Due to sequence divergence in the 3′UTR of *CGB1–CGB2*, the predicted STOP codons for +47 bp *CGB1* and +47 bp *CGB2* differ by seven amino acids. Alternative transcripts +166 bp (not shown in the figure) and +176 bp contain additional 119 and 129 bp sequences identical to the intronic part of all *CGB* genes (black box). The predicted STOP codon for +166 and +176 bp forms is located at position 355 from the transcription start. *Source: Adapted with permission from Refs 20,21.*

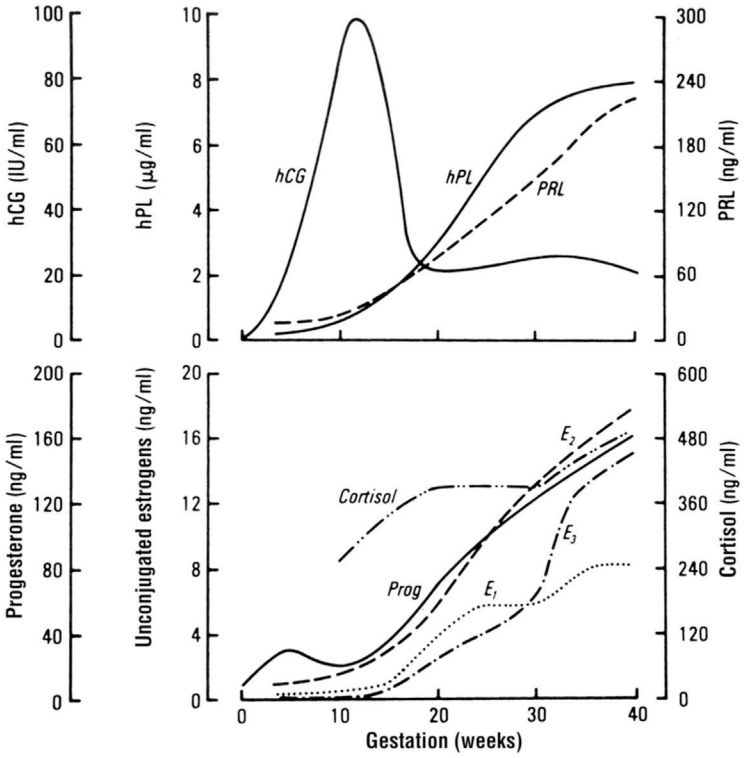

FIGURE 40.3 Serum concentrations of prolactin, human chorionic gonadotropin (hCG), human placental lactogen (hPL), cortisol, progesterone, and unconjugated estrogens during pregnancy. The values have been obtained from several sources in the literature. E_1, estrone; E_2, estradiol; E_3, estriol; PRL, prolactin. *Source: Reprinted with permission from Ref. 26.*

maternal circulation. However, fetal hCG levels are only 3% of those in the mother, whereas levels in amniotic fluid are similar to maternal concentrations during the first trimester, then decline to 20% of that in maternal blood thereafter. Amniotic fluid hCG is thought to originate from direct secretion by trophoblast cells, as well as production by and excretion from the fetal kidney.[27]

It is well established that the profile of hCG in maternal blood is determined by the rate of hCG synthesis and reflects changes in both the number of hCG-producing cells and the synthetic and secretory capacity of individual cells. Thus, the increase in hCG after implantation parallels the increase in trophoblast cell numbers, whereas the decline in hormone production after the first trimester is associated with a decrease in the number of cytotrophoblasts due to fusion of these cells and transformation to the syncytiotrophoblast. The rate of synthesis of hCG and the expression and levels of mRNAs for the α and β subunits, including both type 1 and type 2 βhCG, are greater in trophoblast cells obtained in early than in late gestation.[28]

Maternal blood levels of hCG and other placental-derived proteins, alone or in combination, are altered in and thus may be predictive of several pregnancy complications. For example, in addition to serving as confirmatory of pregnancy, hCG levels are increased in women who develop hypertension and preeclampsia and in Down syndrome, are reduced in early gestation in pregnancies complicated by FGR,[29] and may also serve as a predictor of pregnancy outcome (e.g., preterm birth and fetal demise).[30] Moreover, placental expression of the βhCG genes is reduced in pregnancies with recurrent miscarriage.[21,22] Whereas expression of the βhCG5 and βhCG8 genes is biallelic in healthy pregnancies, monoallelic expression of maternal alleles and hemimethylated gene promoters often characterizes recurrent pregnancy loss.[31] Recent studies have confirmed the existence of more than 70 variants of the βhCG5 and βhCG8 genes.[32] Interestingly, a significant protective effect against miscarriage was associated with two single-nucleotide polymorphisms in intron 2 of both genes and with four variants in the βhCG5 promoter. In contrast, in women with recurrent miscarriage, three nonsynonymous substitutions were identified: a p.Val56Leu mutation in βhCG5 that altered the assembly and functionality of intact αhCG–βhCG heterodimers, a p.Pro73Arg substitution in βhCG8 that altered the conformation of the βhCG subunit, and p.Arg8Trp in βhCG8 that was neutral in a structural–functional context.[32]

Regulation of Synthesis

It is well established that the cAMP–PKA pathway has a pivotal role in stimulating hCG secretion.[33] cAMP, which stimulates PKA and phosphorylation of CREBs essential for binding to CRE in the 5′-flanking region of the hCG genes, increases the mRNA levels of both α and β subunits.[34] Two adjacent *cis*-acting elements that bind a phosphorylated form of CRE-binding protein,[15] GATA-binding proteins,[35] and POU domain class 5 transcription factor 1-modulated ETS (E26)[36] regulate αhCG (CGA) expression by trophoblast. Intracellular cAMP also enhances processing of N-linked oligosaccharide chains, resulting in carbohydrate structures with increased sialic acid content.[37] An elevation in hCG mRNA level is due to an increase in the rate of gene transcription and enhanced stabilization of newly formed RNA.[38] Although activation of the diacylglycerol–protein kinase C pathway by phorbol esters stimulates hCG synthesis, this only occurs after a long lag period following activation of α and β subunit mRNA synthesis. Thus, the physiological role of this pathway remains to be determined, although it has been suggested that synergistic interaction between the PKA and PKC pathways may underpin optimal hCG secretion and release.[5]

Khodr and Siler-Khodr[39] originally showed that the human placenta produced a gonadotrophin-releasing hormone (GnRH) that stimulated hCG production in vitro. Additional studies confirmed placental expression of GnRH receptor and that the receptor was linked to cAMP. However, as previously reviewed by Ogren and Talamantes,[5] numerous other factors alone or in combination have been shown to modulate hCG production and/or mRNA expression by native or transformed trophoblast cells. These include growth factors (e.g., EGF, fibroblast growth factor (FGF), and IGF1), steroid hormones (e.g., cortisol), cytokines (e.g., interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), and TGFβ), other hormones (insulin, oxytocin, and arginine vasopressin), as well as oxygen[40] and agonists of the peroxisome-proliferator activated receptor gamma (PPARγ).[41] Thus, despite intensive investigation, our understanding of the physiological role and potential interaction of the latter factors in regulating the synthesis of trophoblast hCG is incomplete.

Function

The most well-established role of hCG is in the regulation of the maintenance and function of the corpus luteum in early pregnancy. hCG binds to the LH–CG receptor to maintain the life span of, promote luteal angiogenesis within, as well as stimulate progesterone (P_4) and estradiol (E_2) synthesis by the corpus luteum.[42] hCG stimulates luteal P_4 and E_2 production by increasing low-density lipoprotein (LDL) receptor-mediated uptake of cholesterol substrate and increasing the expression of key enzymes, including P450 cholesterol side-change cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and aromatase (P450arom), required for steroidogenesis.[43] A second, clearly

established role of hCG is on fetal testis development and function in the first trimester. hCG secreted into the fetus during 8–12 weeks of gestation stimulates testosterone production by the fetal testes,[44] a process essential for differentiation of the internal duct system and external genitalia in the male fetus. hCG has also been shown to stimulate production of relaxin by the corpus luteum, which may act locally to prevent regression of luteal cells, as well as elicit vascular effects in other organs and peripheral tissues.[45]

As reviewed by Rao,[46,47] however, hCG appears to regulate several processes in addition to gonadal function. Thus, hCG produced by the blastocyst also appears to participate in the process of decidualization[48] and development of endometrial receptivity for implantation, in part by controlling expression of factors (e.g., FGF2) that promote cross-talk between the embryo and endometrium[49] and the β-galactoside-binding protein galectin-3.[50] Recent studies show that hCG amplifies IL1 signaling and responsiveness in endometrial stromal cells, leading to the release of angiogenic factors and induction of angiogenesis, thereby coordinating embryonic signaling with endometrial function to promote embryonic growth (Figure 40.4).[51] hCG may also play a role in promoting immune tolerance during human pregnancy[52] by attracting T regulatory cells to the maternal–fetal interface, increasing the number of T cells and their capacity to secrete suppressive cytokines,[53] and enhancing expression of complement regulatory proteins.[54] In addition, hCG binds to its receptor expressed on villous cytotrophoblasts to enhance fusion of these cells, a process that underpins syncytiotrophoblast formation. Thus, it has been proposed that hCG acts in an autocrine manner to control development of the placenta.[55] hCG also stimulated expression of placental leptin[56] and VEGF,[57] uterine angiogenesis,[58–60] and placental expression of the 11β-hydroxysteroid dehydrogenase (11β-HSD)-2 enzyme controlling cortisol catabolism.[61] The high levels of hCG secreted into the maternal circulation during early gestation are associated with a marked reduction in serum thyroid-stimulating hormone (TSH) concentrations, which rebound when hCG levels decline after weeks 12–14 of gestation.[62] Thus, it has been proposed that hCG binds to the TSH receptor and thereby increases maternal thyroid hormone production.[62] Indeed, women with hyperemesis gravidarum or hCG-producing trophoblastic tumors (e.g., hydatidiform mole or choriocarcinoma) often have extremely high levels of hCG and concomitant transient hyperthyroidism.

Recent evidence also indicates that hCG stimulates proliferation[63] and promotes quiescence[64] of uterine myometrial smooth muscle during the second half of gestation. Thus, hCG reduces the amplitude of oxytocin-stimulated uterine contractions by activating large conductance calcium-activated potassium channels of myometrial cells,[64] and it decreases expression and activity of the phosphodiesterase-5 enzyme (PDE5) controlling hydrolysis and thus intracellular levels of cyclic nucleotides (e.g., cyclic guanosine monophosphate (cGMP)) that regulate contractility.[65] Whether the decline in hCG levels late in gestation and/or the apparent decrease in myometrial hCG receptor expression prior to onset of labor[66] facilitates prostaglandin and oxytocin-induced myometrial contractions remains to be determined.

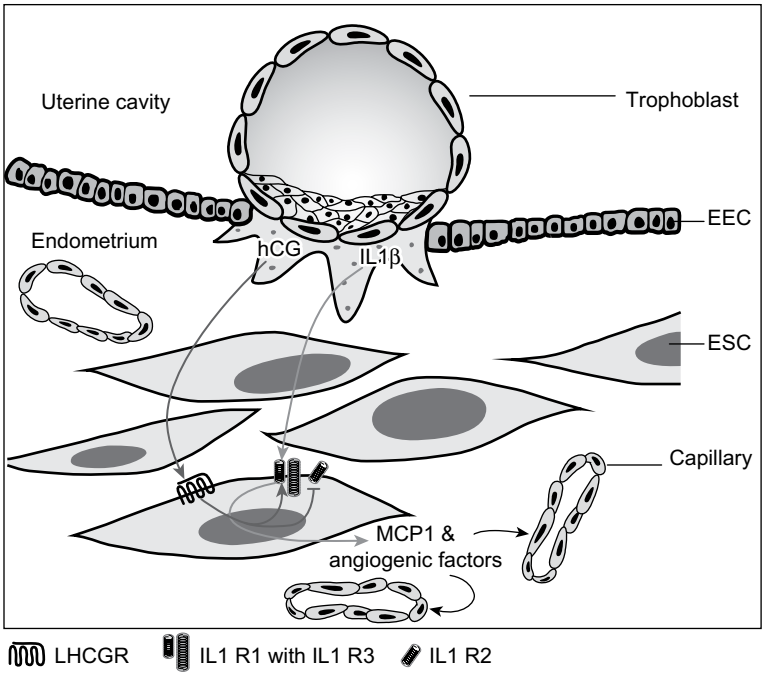

FIGURE 40.4 Hypothesis of hCG and IL1 cross-talk in early pregnancy. Synthesized early by trophoblastic cells, hCG and IL1B act through the endometrial tissue on endometrial stromal cells (ESCs) via their respective receptors (LHCGR, IL1R2, and IL1R1). hCG amplifies IL1 signaling in ESCs, leading to increased cell responsiveness to IL1, the release of angiogenic factors, and the induction of angiogenesis. One of these identified factors is MCP1. EEC, endometrial epithelial cells. *Source: Reprinted with permission from Ref. 51.*

hCG action involves activation of the hCG receptor and induction of cAMP and PKA.[67] Recent studies[68] have shown that cAMP-induced PKA signaling involves binding of the regulatory subunits of PKA to A-kinase anchoring proteins (AKAPs). Such binding ensures specific subcellular compartmentalization of PKA and thus spatial and temporal regulation of PKA signaling events. The AKAPs are composed of a family of more than 50 members that are structurally diverse, but functionally related, proteins that bind to and recruit PKA to locations where it can be accessed by specific regulators and phosphorylate specific substrates. Several AKAPs have been identified in human placenta, including ezrin, AKAP18γ, AKAP350, AKAP70, AKAP95, and AKAP250. The spatiotemporal regulation of PKA signaling by hCG appears to underpin its action (e.g., on placental function).[68] Interestingly, hCG appears to stimulate placental leptin expression through a PKA-independent cAMP and exchange protein activated by cAMP.[69] Moreover, hCG-induced stimulation of the migration and invasion of prostate cancer cells is mediated by activation of extracellular-regulated kinase 1 and 2 (ERK1/2) and matrix metalloproteinase 2 (MMP2).[70] Finally, although hCG and LH act on the same receptor, recent evidence indicates that intracellular signaling may be quantitatively and/or qualitatively different (e.g., hCG is a more potent stimulator of cAMP production).[71]

HYPERGLYCOSYLATED hCG (hCG-H)

Recent evidence indicates that there are five independent hCG variants, each with the same amino acid sequence and differing in the nature and extent of posttranslational processing, which influences protein folding and thus biologic action.[7] The two hCG molecules produced by the placenta are hCG synthesized by the syncytiotrophoblast and hCG-H synthesized by EVTs.[72–74] Additional studies confirmed that EVTs expressed αhCG and βhCG mRNA and that expression was modulated by agonists of PPARγ.[41] The pituitary produces what is now termed sulfated hCG. Malignant cells produce hCGβ and hyperglycosylated hCGβ, which are not linked with the α subunit.[75] A summary of the properties of these five independent variants of hCG is shown in Table 40.1.

Maternal serum levels of hCG-H are actually higher than those of hCG during the first 6 weeks of gestation, peak at about weeks 10–14, and decline thereafter. Several forms of glycosylated hCG, including hCG-H, have been detected in maternal serum, including a specific variant originally termed invasive trophoblast antigen,[75] which is highly expressed in Down syndrome. hCG-H has a molecular weight of 42,800 due entirely to enhanced sugar content (Table 40.1), which leads to incomplete folding of the protein and exposure of sequences otherwise hidden, most notably the cryptic central cysteine knot domain.[7] This structure has also been identified in a number of factors that comprise a cystine knot superfamily that includes VEGF and TGFβ.[76] Accordingly, hCG-H has been shown to bind to and antagonize TGFβ receptors on cytotrophoblast cells, a process underpinning implantation of the blastocyst as well as stimulating angiogenesis through TGFβ receptor activation.[75,76] It has been suggested that hCG-H works with hCG to control differentiation and fusion of new cytotrophoblast cells to form the syncytiotrophoblast and thus placental growth,[73] as well as promote trophoblast migration and invasion in vitro.[7,77] Thus, it has been suggested that a deficiency of hCG-H causes incomplete blastocyst implantation, as well as miscarriage, and thus is a marker for failing pregnancy.[75] Although additional studies remain to be performed, screening of hCG-H alone and/or in conjunction with other factors of placental origin may be critical for early diagnosis of patients likely to exhibit pregnancy complications, notably Down syndrome and preeclampsia.[75]

Placental Lactogen

Structure and Expression

PL, also designated chorionic somatomammotropin (CS), first partially purified in the early 1960s,[78–80] is a member of the growth hormone (GH) family, which also consists of pituitary GH (GH-N) and placental GH variant (GH-V).[81–83] Members of the GH-PL gene cluster are located on chromosome 17 and are very similar in structure.[84] Expression of the GH gene cluster is regulated by a locus control region,[85] which contains hypersensitive sites. These short regions of chromatin become sensitive to DNase cleavage when transcription factors bind to DNA and displace histones within the hypersensitive site.[86] The hypersensitive sites are placental and pituitary specific, and they recruit histone acetyltransferases and deacetylases, which link to transcriptional activation within the GH promoter. Differentiation of placental cytotrophoblasts into the syncytiotrophoblast results in histone methylation of the DNase I-hypersensitive sites III, IV, and V (HSIII–HSV)[87] and acetylation of histones encompassing the entire GH cluster region.[86]

Human PL (hPL) is a nonglycosylated single-chain polypeptide with 191 amino acids and a molecular weight of 22,279. There is over 96% amino acid homology between PL and GH. Two disulfide bonds stabilize the secondary and tertiary structure of PL. Three genes, CSA (designated CSH1), CSB (aka CSH2), and CSL, encode PL and exhibit 94–98% sequence homology in their coding and flanking regions. The CSA and CSB genes encode PL protein differing by a single amino acid,

TABLE 40.1 Properties of the Five Independent Variants of Human Chorionic Gonadotropin (hCG)

Parameter	hCG	Sulfated hCG	Hyperglycosylated hCG	hCGβ	Hyperglycosylated hCGβ
Source of synthesis	Syncytiotrophoblast	Gonadotrope	Cytotrophoblast	Advanced malignancy	Advanced malignancy
Mode of action	Endocrine	Endocrine	Autocrine	Autocrine	Autocrine
Total molecular weight	37,180	36,150	42,800	23,300	27,600
Site of action	LH–hCG receptor	LH–hCG receptor	TGFβ antagonism	TGFβ antagonism	TGFβ antagonism
Peptide molecular weight	26,200	26,200	26,200	16,000	16,000
Molecular weight sugars	10,980	9950	16,600	7300	11,600

Modified from Ref. 7.

while the secreted protein products of the genes are identical.[88–90] The CSL gene contains a G-to-A transversion at the splice site, which prevents pre-mRNA processing and thus expression of CSL protein of the same size as PL. The expression of placental CSA mRNA increases 30-fold and CSB mRNA 10-fold between early and late gestation,[91] suggesting that the CSA gene is more transcriptionally active as placental syncytial development occurs.[92,93] Approximately 90% of CS mRNA is encoded by the CSA gene in the first and second trimesters of human pregnancy.[89,91,94]

Elegant studies have recently been conducted to elucidate the structural events that govern utilization of the transcription start sites of the CSA and CSB genes and the sequence requirements for efficient CS expression in vivo. A 241 bp region located downstream of the CSB gene, which contains hyperacetylated histones H3 and H4, enhances promoter activity in human placental cells.[84,87] Moreover, transcription enhancer factors (TEF), including TEF1 and TEF5, bind to an enhancer element to confer placental-specific CSB expression.[95,96] The difference in transcriptional activity between CSA and CSB enhancer regions has been traced to one or two nucleotide differences in repressor and de-repressor elements.[97,98] Moreover, members of the CCAAT–enhancer-binding protein (C/EBP) and ETS family of transcription factors also differentially modulate CSA and CSB enhancer activities.[99] C/EBP and ETS binding in the CSA enhancer may be significant, since ETS signaling appears to play an important role in development of the placenta.[100] Moreover, by regulating promoter–enhancer complexes and/or chromatin modification, ETS and C/EBP members also may contribute to the expression of CS in the placenta and its lack of expression in the pituitary.[99] The promoter regions of the CSA and CSB genes also bind the transcription factor specificity protein 1 (SP1), which is necessary for transcriptional activation of these genes.[95]

Production

PL is synthesized within the villous placenta, and differentiation of villous cytotrophoblasts into the multinucleated syncytiotrophoblast during the first trimester of human pregnancy results in a striking exponential upregulation of PL production (Figure 40.3).[101,102] The maternal serum levels of PL correlate directly with placental mass. Near term, levels of hPL are approximately 1000-fold higher in maternal (5–10 μg/ml) than in fetal (20–30 ng/ml) circulation, suggesting that PL is differentially secreted from the syncytiotrophoblast into the maternal compartment. The daily production of hPL approximates 1 g, an amount exceeding that of any other protein hormone produced by the placenta. Human PL is stored in and released via exocytosis from Golgi, and the half-life of PL is approximately 20 min.[103]

Regulation of Synthesis

Because PL appears to have an important role in modulating intermediary metabolism within the mother, there has been extensive investigation to determine the effects of components of intermediary metabolism on PL synthesis. Prolonged fasting in the human results in a significant increase in maternal plasma PL levels.[104] Although a decrease in maternal hPL levels with hyperglycemia has been reported,[105] other in vivo and in vitro studies have not shown an effect of glucose on PL secretion.[106,107] Moreover, conflicting results of the relationship of insulin and triglycerides to PL secretion have also been shown in cultures of term placental cells.[108–110] It appears, therefore, that prolonged fasting and hyperglycemia alter the secretion of PL, while short-term alterations in glucose and related intermediary metabolites have little impact on PL release.

Handwerger and colleagues have shown that physiological levels of high-density lipoprotein (HDL), as mediated by apolipoproteins AI and AII, stimulate PL secretion by human trophoblast cultures.[111–114]

Moreover, GH-N[115] and GH-V, which bind to the GH receptor expressed in the placenta[116]; EGF[117]; IGF-I[118]; and the transcription factors PPARγ[119] and activator protein 2[120] stimulate PL secretion in cultures of human placental cells, although the regulatory effects of the growth factors may be related to transformation of cytotrophoblasts to the syncytia. Studies of the role of steroid hormones on placental PL secretion have yielded disparate results. Thus, glucocorticoids, E_2, and P_4 have either increased or no effect on PL release in vivo or in vitro by human placental explants.[110,117,121,122] GH-releasing hormone (GHRH) and the dopamine, acetylcholine, and β-adrenergic neurotransmitters that are present in the placental milieu stimulated, inhibited, or had no effect on PL release in vitro.[123–125] Arachidonic acid and cAMP stimulated the in vitro secretion of PL,[126–128] while oxygen induced expression of the GH cluster genes.[129]

Thus, despite extensive study, a clear picture of the physiological regulation of PL secretion has not been developed. This problem may reflect the widely different in vitro and in vivo experimental approaches that have been employed in this area of placental physiology.

Function

Since the structure of PL is very similar to that of GH and prolactin (PRL), and PL binds with high affinity to prolactin and GH receptors, it is not surprising that PL has effects on intermediary metabolism, growth, and lactogen function. In vitro studies showed that PL stimulated human pancreatic islet beta cell replication and insulin secretion,[130–132] and that overexpression of PL in beta cells increased mitosis and insulin production[133–135] and protected against the development of diabetes in streptozotocin-treated mice.[134,136] Knockout of the prolactin receptor, which is expressed in islet beta cells,[137,138] reduced beta cell mass, proliferation, and insulin secretion and impaired glucose clearance but not insulin sensitivity,[139,140] indicating that lactogen signaling is necessary for pancreatic beta cell development and function. PL and prolactin induced beta cell expression of cyclins A, B, and D and decreased expression of Fox01, FoxM1, cyclin-dependent kinase inhibitors, and the transcriptional coactivator menin that targets the latter inhibitors.[141–143] PL also stimulated the production of growth-promoting serotonin[144] and enhanced beta cell survival[136] in cultures of rodent pancreatic islets. In cultures of beta TC 1 (βTC1) insulinoma cells and human pancreatic islets, PL but not GH promoted cell survival and insulin secretion by rapid activation of the protein kinase B (Akt)–mitogen-activated protein kinase (MAPK) signaling pathway, phosphorylation of Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs), as well as expression of the homeobox pancreatic duodenal homeobox 1 (PDX1) transcription factor.[132,145] The parallel increase in circulating PL levels and pancreatic beta cell replication during human and rat pregnancy[146,147] is consistent with a role for the lactogens in promoting maternal beta cell replication. Since lactogenic hormones counteract the inhibitory effects of glucocorticosteroids on expression of pyruvate dehydrogenase,[148] which is important to pyruvate metabolism and flux into the Krebs cycle, Freemark and coworkers[149,150] have suggested that lactogenic hormones may maintain beta cell mass and function under conditions of stress and nutrient deprivation to protect against the development of gestational diabetes.

In contrast to these recent findings, earlier studies have shown that PL either increased[151] or had no effect[152] on insulin secretion in vivo and decreased insulin sensitivity in the liver of rodents.[153] Moreover, although PL has been shown to stimulate glucose uptake and oxidation during the fed state consistent with its effects on insulin secretion, PL reduced insulin sensitivity and induced glucose intolerance during fasting in the presence of elevated levels of prolactin and glucocorticoids at mid- to late pregnancy.[151,154] The latter effects, along with an increase in lipolysis,[155] may ensure the utilization of free fatty acids as a source of energy for the mother, while sparing glucose as a primary source of energy for the fetus. Since increased maternal lipolysis and fatty acid re-esterification occur in association with the elevation in PL during advancing human pregnancy,[156] these effects appear to be physiologically relevant. More recent studies, however, have not confirmed the direct lipolytic effects of lactogenic hormones,[157] and thus the role of these hormones in maternal lipid metabolism and insulin resistance is unclear. It also appears that PL and/or prolactin may play a role in maternal hyperphagia and weight gain during pregnancy,[149,158] possibly by inducing hypothalamic neuropeptide Y (NPY) expression.

There is considerable evidence that PL promotes fetal growth, since PL stimulated amino acid uptake and thymidine incorporation by human fetal fibroblasts and skeletal muscle,[159,160] tissues that express PL-binding sites. Because expression of IGF1 and IGF-binding protein (IGF-BP) was simultaneously increased by PL administration,[161] the regulatory effects of PL on fetal tissue growth may be mediated by IGF1. Finally, PL also stimulated proliferation of epithelial cells[162] and ductal growth[163] in the human mammary gland and lactogenesis in laboratory animals.[164]

Growth Hormone Variant

Growth hormone variant (GH-V), first described in 1985,[165] is a glycosylated protein that contains 191 amino acids and differs from GH-N by only 15 amino acids. The GH-V gene undergoes alternative splicing producing a second protein designated GH-V2. GH-V2 accounts for approximately 5% in the first trimester and 15% in the

third trimester of the placental GH-V gene mRNA transcripts. However, hGH-V and hGH-V2 mRNAs account for less than 0.05% of total placenta mRNA at term. GH-V is synthesized by the syncytiotrophoblast, and maternal circulating levels progressively increase, reaching a maximum of 20–60 ng/ml in the third trimester of human pregnancy.[166] Although GH-V is not detectable in the fetus, GH-N is present in relatively high levels, 20–30 ng/ml, in fetal serum.

Regulation of Synthesis and Function

Relatively little is known about the regulation of GH-V synthesis, although triiodotyrosine[167] and the transcription factor AP2[120] increased, while GHRH had no effect[168] on GH-V expression or secretion by BeWo choriocarcinoma cells or primary placental cells. The GH-V promoter is transactivated by the transcription factors myocyte enhancer factor 2 and FoxF1, an effect that may involve coactivation.[169] GH-V binds to the prolactin and GH receptors, and the affinities of GH-V and GH-N for the GH receptor are similar. GH-V exhibited somatotropic[170] and metabolic[171] effects with potency approximating that of GH-N. GH-V is also considered a major mediator of insulin resistance during pregnancy, via its modulatory action on IGF1 expression and insulin action in transgenic mice.[172]

Physiological Relevance and Clinical Correlations of PL and GH-V

Disruption of fetal development (e.g., FGR) during human pregnancy is associated with lower placental PL and GH-V levels or transcripts.[94,149,173,174] In contrast, placental CSA and CSB but not GH-V, mRNA transcript expression, and PL levels were greater in pregnancies with large for gestational age babies.[86,94,175] Thus, PL and GH-V appear to be differentially regulated and expressed in association with altered fetal growth, possibly as a result of polymorphisms or epigenetic modifications in the locus control region, alternative allelic composition of the promoter affecting binding of regulatory transcription factors, or changes in placental trophoblast differentiation.[87,176] It has been postulated that the differential regulation of PL and GH-V in pregnancies with small and large for gestational age babies may provide adaptive maternal and fetal benefits, whereby alterations in PL and GH-V induced by changes in placental sufficiency change the production of insulin and/or IGF1, leading to decreased or increased fetal growth.[177]

Of a total of 11 human pregnancies in which PL was not detectable in maternal plasma, five had low newborn birth weights in the 7th percentile.[149] Deletion of PL and GH-V was associated with either FGR or unaltered fetal growth.[177] As suggested by Freemark,[149] the disparate results on birth weight may reflect compensatory upregulation of the expression of members of the GH family and/or other growth factors in response to PL or GH-V deletion. Decreased plasma levels of PL also occur in pregnancies complicated by preeclampsia and other pathologic conditions, including diabetes mellitus and hypertensive vascular disease,[178–180] most likely because of the decrease in placental mass associated with these clinical conditions. Freemark and colleagues[158] have created a prolactin receptor knockout–GH-deficient mouse model to study the roles of the lactogens and somatotropic hormones in perinatal and postnatal growth and metabolism. Although body weights and plasma glucose were normal in single-mutant mice, double mutants exhibited growth retardation and hypoglycemia, suggesting that lactogens and somatogens act in concert to promote perinatal growth and glucose metabolism.

In summary, despite the effects of PL shown in vitro or in vivo, several studies have shown that women with very low or undetectable levels of hPL (because of partial or complete deletion of the CSA and CSB genes)[181,182] maintained pregnancy and exhibited normal glucose tolerance, insulin sensitivity, lactation, and newborn birth weight.[183–185] The disparate effects of PL on these physiological endpoints may reflect the wide range of experimental approaches, the physiological conditions of and use of nonpregnant as well as pregnant subjects, and variation in the doses and preparations of PL employed in these studies. In addition, PL may act in concert with prolactin, GH-N, GH-V, as well as other growth factors (e.g., IGFs) in regulating metabolic adjustments, pregnancy maintenance, and fetal growth during advancing human pregnancy.

Placental Growth Factors

IGF-I and -II

The IGFs are members of a family of peptides related structurally to insulin. IGF-I and IGF-II share >60% sequence homology, have a molecular mass of approximately 7600 kDa, and, like insulin, are composed of α and β chains connected by disulfide bonds.[186–188] The gene for IGF-I is located on chromosome 12 (12q22–24.1), and transcription is governed by two promoters, one adjacent to exon 1 and the other 5′ to exon 2. The IGF-I gene also has several polyadenylation sites within exon 6. Both promoters regulate transcription at several sites, but they lack "TATAA"-, "CCAAT"-, or "GC"-rich regions. Moreover, the mRNAs derived from promoters 1 and 2 are processed via alternative splicing, which, along with alternative polyadenylation, leads to several IGF-I mRNA transcripts.[189] The gene for IGF-II is located adjacent to the H19 gene on chromosome 11p15.5, and both genes share the same regulatory transcription factors and are imprinted. Genomic imprinting (i.e., the silencing of one copy of an autosomal gene and

expression of the other) is regulated by epigenetic mechanisms, including DNA methylation. Imprinted genes that are paternally expressed (maternally imprinted), such as IGF-II, promote fetal growth, whereas those that are maternally expressed (e.g., H19) act as growth suppressors.[190] Two regions of allele-specific methylation of the human IGF-II gene have been identified.[191] Recent study has shown that loss of imprinting of the IGF-II gene, which as expected was negligible in placentas of women delivering normal-weight babies, was extensive in the placentas of pregnancies with FGR.[192] The authors suggested that loss of IGF-II imprinting in FGR likely contributes to the placental dysfunction that is a major component of the pathogenesis of FGR. However, the factors regulating DNA methylation and silencing of the placental IGF-II gene, and whether loss of silencing is the result or cause of FGR, remain to be determined.[193]

Following release into the circulation or the intercellular space, IGF-I and IGF-II are bound to binding proteins (IGF-BP) that modulate the availability, as well as influence the stability and thus control the activity, of the IGFs. Six IGF-BPs with high affinity for the IGFs have been identified in serum following production by and release from numerous tissues, notably the liver. During pregnancy, the mRNAs for all of the IGF-BPs are expressed by the decidua, trophoblast, and other cells within the placental basal plate. However, IGF-BP3 is the predominant maternal serum IGF-binding protein, binding 70–80% of total serum IGF-I and IGF-II,[194] and levels of IGF-BP3 as well as IGF-BP1 increase with advancing gestation.[195] The physiologic actions of IGFs are controlled by binding to two cell membrane receptors, an IGF-I receptor and an IGF-II receptor.[196] The IGF-I receptor is a heterotetramer that exhibits ligand-activated tyrosine–kinase activity and binds IGF-I > IGF-II > insulin. In contrast, the IGF-II receptor is a monomer that binds IGF-II > IGF-I, as well as mannose-6-phosphate, but does not bind insulin. Site-directed mutagenesis has shown that the IGF-I and IGF-II receptors have two separate binding surfaces and that cross-bridging to the two IGF receptor halves involving residue Glu12 is crucial to receptor activation.[197,198] Both IGF-I and IGF-II receptor signaling involve receptor autophosphorylation and activation of phosphatidylinositol 3-kinase (PI3K), ERK–MAPK, and/or Akt.[199]

Both IGF-I and IGF-II are expressed in the human placenta in addition to other tissues (e.g., liver and adipose).[200,201] The mRNA for IGF-I is detected primarily in the syncytiotrophoblast, whereas IGF-II mRNA is expressed in cytotrophoblasts, the syncytiotrophoblast, EVTs, and placental fibroblasts.[200,202] Placental IGF-I and IGF-II mRNAs are detected as early as week 8 of human pregnancy,[203] and although placental IGF-I mRNA expression appears to decrease between mid- and late gestation,[202,204] there is a marked increase in maternal serum IGF-I and a slight, albeit significant, increase in IGF-II levels with advancing human pregnancy.[205,206] The IGF receptors are localized within the syncytiotrophoblast, as well as the cytotrophoblast and villous stroma.[203,207–209] Although our understanding of the factors controlling placental IGF mRNA expression remains incomplete, the site and level of expression of the mRNAs for, as well as maternal serum levels of, IGF-I and GH-V are positively correlated regardless of pregnancy complication and gestational age.[210] These findings, plus the observation that villous trophoblasts and EVTs express the GH receptor that binds GH-V,[211] suggest that GH-V is a primary regulator of placental IGF-I.[205] Although not secreted into the fetal circulation, the maternal levels of total GH-V, as well as GH-V not bound to GH-binding protein, increase from week 7 until term. Several studies have shown that the rise in maternal serum GH-V is associated with an increase in maternal IGF-I levels, particularly after week 24 of gestation, and highly correlated with fetal growth.[205,206,212] Moreover, placental mRNA expression as well as serum levels of both GH-V and IGF-I are decreased in pathologic human pregnancies (e.g., those with FGR),[166] further supporting a role for GH-V in promoting fetal growth via regulation of IGF-I and presumably actions on placental systems controlling substrate supply to the fetus. Maternal levels of IGF-II also are comparably decreased at weeks 28 and 36 of gestation in FGR pregnancies.[166] However, maternal serum levels of total and free or unbound GH-V were not significantly altered, while maternal and fetal serum levels of IGF1 were lower and IGF-BP3 levels higher in pregnancies complicated by type 1 diabetes.[213] Thus, it would appear that the increase in insulin requirements during pregnancy in type 1 diabetics is not related to GH-V levels.[214]

Gene deletion and other studies have confirmed the importance of IGF-I and/or IGF-II in promoting cell proliferation and survival and placental and fetal growth and development.[188,215–217] For example, mice with deletions of IGF-I and/or IGF-II have pups that are 30–60% smaller than normal.[218,219] However, although neonates of IGF-II knockouts survive and exhibit normal growth after birth, postnatal growth is compromised in IGF-I-deleted animals, the majority of which die before reaching adulthood.[188] While placental growth was normal in IGF-I knockouts, placentas were small and hypoplastic in IGF-II-null animals.[220] Moreover, administration of IGF-I to normal or growth-restricted fetal sheep and guinea pigs increased or restored growth and maturation,[221–224] apparently via upregulating placental nutrient transporters via activation of mammalian target of rapamycin (mTOR),[225] which mediates maternal nutrient availability for fetal growth.[226] In addition, severe FGR was noted in infants with a heterozygous missense mutation,[227,228] or a homozygous mutation within the

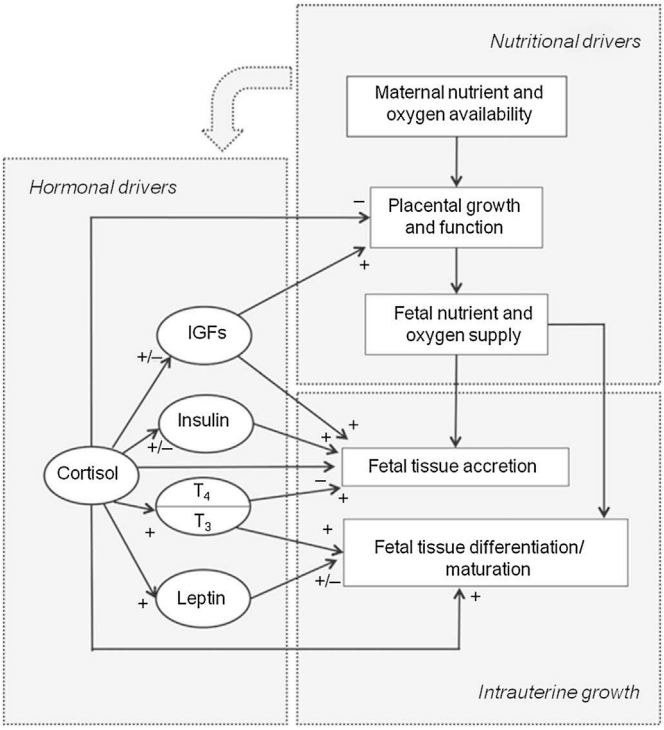

FIGURE 40.5 A schematic diagram showing the interactions of hormonal and nutritional drivers in the control of intrauterine growth. *Source: Reprinted with permission from Ref. 217.*

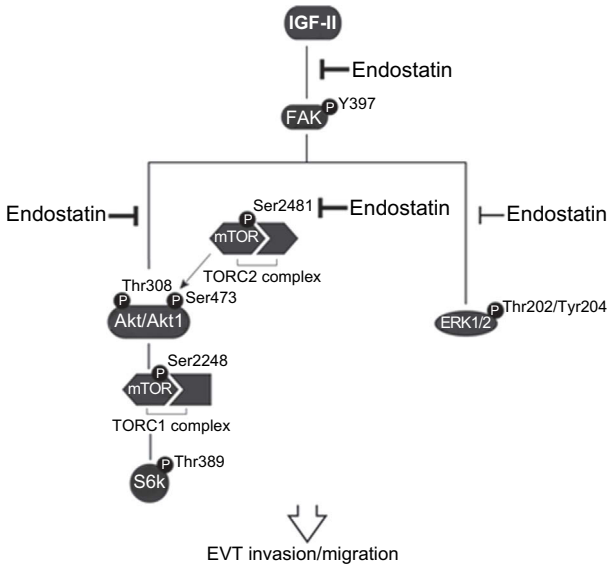

FIGURE 40.6 Proposed model of endostatin-mediated suppression of trophoblast motility. Endostatin exerts broad effects on IGF-II-mediated signaling in human trophoblasts by affecting phosphorylation of FAK–Akt–mTOR–S6K and, to a lesser extent, ERK1/2. Of note, endostatin interferes with both mTOR complexes, TORC1 and TORC2, thereby affecting activation as well as downstream signaling of Akt. However, suppression of the Akt phosphorylated residue at Thr308 suggests additional endostatin targets upstream of Akt, such as FAK. Finally, its main suppressive role in the context of trophoblast invasion was found to be dependent on the expression of Akt1. *Source: Reprinted with permission from Ref. 237.*

kinase[229] or the extracellular fibronectin III[230] domains of the IGF-I receptor. Moreover, null mutation of the Akt1 component of the IGF receptor signaling pathway, as with IGF-II-null mutation, compromised placental, fetal, and postnatal growth.[231] These and other studies support the suggestion that IGF-I has a relatively greater role in fetal growth and development, whereas IGF-II is more important for placental development. However, IGF-I and IGF-II do not act alone in this regard, and highly complex regulatory interrelationships between the IGFs and insulin, thyroid hormone, cortisol, and leptin, as well as nutritional drivers, appear to control placental–fetal growth (Figure 40.5).[217]

In addition to regulating fetal and placental growth, in vitro studies with primary human placental trophoblast and BeWo cells have shown that IGF-I and IGF-II stimulated trophoblast proliferation and survival,[232–234] as well as migration and vessel invasion.[235,236] IGF-BP3 inhibited IGF-I- and IGF-II-induced proliferation of placental trophoblasts by signaling through the TGFβ receptor.[233] Moreover, endostatin, a biologically active fragment of collagen XVIII, inhibited IGF-II-induced placental trophoblast migration and invasion by interfering with the activation of ERK1/2, Akt–mTOR–p70 S6 kinase, and focal adhesion kinase (FAK) (Figure 40.6).[237] Furthermore, although IGF-I did not alter placental artery tone, it reduced the vasoconstrictory effects of a thromboxane mimetic on myometrial arteries,[238] indicating that IGF-I may also modulate delivery of maternal blood to the placenta. Thus, along with their anabolic effects, the IGFs may also promote fetal–placental growth by expanding the placental syncytium and remodeling the uterine arteries to promote maternal–fetal exchange.

TGFβ Super Family: TGFβ

TGFβs are members of a super family of cytokines that includes activins, inhibins, bone-morphogenic proteins, and so on.[239,240] Three isoforms of TGFβ exist, TGFβ1, TGFβ2, and TGFβ3, which are encoded by three different genes. The human placenta expresses mRNAs for TGFβ1 predominantly in the villous placenta, TGFβ2 primarily in the decidua, and TGFβ3 in the villous trophoblasts and EVTs.[241] TGFβ proteins are secreted into the maternal circulation or locally in the maternal–fetal interface, and they are thought to elicit multiple effects on placental function. The TGFβ family members exert biologic actions by binding to the type 2 TGFβ receptor, which initiates interaction with the type 1 TGFβ receptor, forming a dimer that activates receptor serine–threonine kinase activity and induces phosphorylation of the downstream transcription factors SMAD2 (Sma- and Mad-related protein 2) or SMAD3.[242] Both the type 1 and type 2 TGFβ receptors are expressed in human placenta, particularly early in gestation.[243] Because knockout of

either receptor results in severe FGR, it has been suggested that the TGFβ super family plays a role in fetal growth, either directly or via control of placental function.[244] However, placental expression and maternal serum and umbilical cord concentrations of TGFβ1 increase with advancing normal human gestation,[245,246] and they either increase[247] or are unaltered[248] in human pregnancies with FGR; thus, the role of TGFβ1 in fetal growth remains controversial.[209]

In vitro studies have shown that TGFβ1 and TGFβ3 inhibit EVT invasion capacity.[209,249] TGFβ (produced by the decidua) as well as the TGFβ-binding proteoglycan decorin (also of decidual origin) act in a paracrine manner to suppress EVT invasion.[250] The expression and inhibitory effect of TGFβ3 on EVT invasion are enhanced by hypoxia-inducible factor 1 alpha (HIF-1α).[251] TGFβ also mediates the ability of IGFBPs to suppress trophoblast invasive capacity.[252] TGFβ1 and TGFβ3 are elevated in placentas of preeclamptic women,[244,253] and inhibition of TGFβ3 restores the invasive capacity of EVTs isolated in late gestation from placentas of women with preeclampsia.[245,254] However, because studies using placental cells that have been transformed or isolated from women with preeclampsia cannot test cause–effect, it remains to be determined whether the TGFβs play a role in normal placental development and/or become important later in gestation in exacerbating placental dysfunction and thus the complications of diseases characteristic of pregnancy pathologies (e.g., preeclampsia).

TGFβ Super Family: Activin and Inhibin

Activin and inhibin are disulfide-linked heterodimeric or homodimeric protein members of the TGFβ super family originally isolated from gonadal tissue. Activins and inhibins are composed of two different β subunits (β_A and β_B), each encoded by a single gene. The β subunits dimerize, forming activin A (β_A–β_A), activin B (β_B–β_B), and activin AB (β_A–β_B). Inhibins are composed of an α subunit and one of two β subunits (β_A or β_B), giving rise to two functional glycoproteins, inhibin A (α–β_A) and inhibin B (α–β_B). Activin A and inhibin A are expressed in the decidua, syncytiotrophoblast, cytotrophoblast, and fetal membranes.[255] Activin A and inhibin A, as well as follistatin, are the primary placental products formed and secreted into the maternal circulation, and levels of all three increase with advancing gestation and become negligible postpartum.[256] At the cell membrane, activins interact with serine–threonine kinase type 1 and type 2 receptors. Activin bioavailability is blocked by the sequestration of activin by follistatin.

Activin A and inhibin B act in a paracrine–autocrine manner to regulate cell proliferation and differentiation processes in several tissues, including reproductive tissues.[257] Because activin receptors are expressed in the trophoblast, particularly during the first and second trimesters, in villous vascular endothelial cells and fetal membranes late in gestation, and activin stimulates prostaglandin release from amnion cells, roles for activin in placental differentiation, EVT invasion, vascular adaptation of pregnancy, and labor onset have been proposed.[258] Of particular interest is the role of activin A and activin B in modulating inflammatory processes and immunity during pregnancy by regulating the function (e.g., interleukin production) of neutrophils, monocytes, and lymphocytes[259] within gestational tissues.[260] Cross-talk between Toll-like receptor 4 and activins has been shown in animal models of sepsis.[261] Both activin A and inhibin B also differentially regulate expression of human uterine MMPs, and thus cellular remodeling processes involved in decidualization and trophoblast migration and invasion.[249,262] Rosenberg et al.[263] have recently shown that activin A levels declined and inhibin A levels rose in amniotic fluid between normal middle and late human gestation, activin A levels were markedly elevated in women with intraamniotic infection or inflammation independent of amniotic membrane rupture, and activin A was upregulated by the addition of lipopolysaccharide to amniochorion explants. These authors concluded that activin A and inhibin A are dynamic participants in the biological process linked to intraamniotic infection- or inflammation-induced preterm birth. Maternal serum levels of activin A and inhibin A are increased in women with preeclampsia,[264,265] as well as in patients with Down syndrome, those with FGR, and those who deliver prematurely.[258,266] The increase in activin A with preeclampsia may result from the elevation in oxidative stress[267] that accompanies this disease. The combination of maternal serum activin A, inhibin A, and PlGF levels and ultrasound assessment of the uterine artery pulsatility index are predictive of preeclampsia.[268]

Epidermal Growth Factor

EGF, a polypeptide hormone originally isolated from the salivary glands, is a member of the EGF super family composed of heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen, neuregulins1–6 and TGFα. Each of the EGF members activates a family of four receptors, ErbB1–4, also termed HER1–4.[269,270] Receptor activation is very complex and is controlled by the spatiotemporal expression, posttranslational processing, and ectodomain shedding of the ligands, the latter being critical to the interreceptor cross-talk that must be coordinated in response to multiple stimulants.[269]

EGF receptors are expressed in the EVTs and villous cytotrophoblast and syncytiotrophoblast, as well as the decidua, of the human placenta, with expression elevated early and declining with advancing gestation. Maternal serum levels of EGF are also highest in the first trimester and decline with advancing gestation. That EGF and its

receptors play a role in placental development and function is supported by studies showing that pups of mice deficient in EGF are growth retarded, homozygous HER-null mice die in utero due to abnormal placental development, intraamniotic infusion of EGF normalized fetal weight in a rabbit model of FGR, and the EGF receptor is altered in human placentas of pregnancies complicated by FGR.[209] In addition, EGF has been shown to promote trophoblast proliferation and differentiation and inhibit trophoblast apoptosis induced by reactive oxygen species or hypoxia by activating, respectively, PI3K or p38 MAPK and phosphorylation of the B-cell lymphoma 2 associated death promoter.[271,272]

HB-EGF is expressed throughout the course of human gestation in the placenta, including the EVTs and villous cytotrophoblast and syncytiotrophoblast, as well as the decidua.[273] Expression of HB-EGF is particularly high in first-trimester placenta and decidua, although much lower in the syncytiotrophoblast. Interestingly, compared with high expression of HB-EGF in placentas from women who delivered at term or 4–8 weeks prematurely, HB-EGF mRNA and protein expression was negligible in late gestation in placentas from pregnancies complicated by preeclampsia and FGR.[274] The latter pregnancy complications are thought to reflect shallow trophoblast invasion of uterine spiral arteries, and in vitro studies using a variety of cell lines have shown that HB-EGF, as well as EGF, can modulate EVT proliferation, migration, and/or invasion.[273] EGF induces an EVT-invasive phenotype, effects blocked by inhibition of tyrosine kinase activity of HER1, which is expressed in EVTs.[275] Expression of HB-EGF, but not EGF or other EGF-like growth factors, was rapidly upregulated in immortalized EVT cells cultured in a hypoxic (i.e., 2% O_2) environment, and cell survival was dependent on HB-EGF expression and receptor signaling.[276] Moreover, because the level of HB-EGF that was cytoprotective was similar to that released into conditioned medium by first-trimester human trophoblast cultured at 2% oxygen, HB-EGF may play a physiologic role in EVT survival. Interestingly, HB-EGF-mediated signaling also reduced trophoblast apoptosis at term. Therefore, it is possible that the absence of placental HB-EGF expression in preeclampsia may underpin the high incidence of apoptosis noted in these placentas. However, the factors regulating HB-EGF expression in normal and abnormal pregnancy (e.g., preeclampsia) have not been elucidated.

Vascular Endothelial Growth Factor

The VEGF super family is composed of VEGF-A (known as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PlGF. VEGF is encoded from a single gene organized as eight exons separated by seven introns, and alternative exon splicing results in the generation of isoforms composed of (after signal sequence cleavage) 121, 145, 165, 189, and 206 amino acids. VEGF is a potent endothelial cell mitogen and anti-apoptotic factor that stimulates vascular permeability and consequently the formation of new blood vessels via angiogenesis.[277–280] Although new vessel growth and maturation are highly complex and coordinated processes requiring sequential activation of a series of receptors by numerous ligands, VEGF signaling represents a critical early rate-limiting step. Thus, inactivation of a single VEGF allele in mice resulted in significant vascular defects in embryonic tissues and organs, as well as embryonic lethality.[281] Native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa and the heparin-binding domain provides essential stimulatory cues for angiogenesis. Elimination of this domain in $VEGF_{165}$ results in loss of mitogenic activity, and thus with respect to angiogenesis $VEGF_{165}$ is considered the most physiologically active isomer.

VEGF binds to two structurally related transmembrane tyrosine kinase receptors, the fms-like kinase (Flt-1, or type 1) and kinase-insert domain (KDR/Flk1, or type 2) receptors (Figure 40.7).[280] Both receptors are required for vascular development since homozygous Flt-1 or KDR–Flk-1 defective mice die in utero.[282,283] VEGF-A

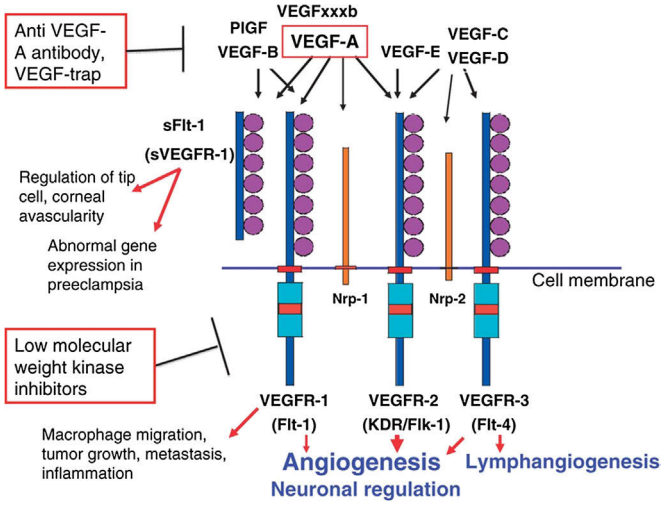

FIGURE 40.7 The VEGF–VEGFR system and its involvement in various physiological and pathological processes. VEGF-A, particularly VEGF165, plays a central role in angiogenesis. VEGFxxxb has recently been reported to act as a natural antagonist to VEGF-A. Three receptors and one soluble form of the VEGFR family members are highly conserved in most vertebrates, except for fish. Major pro-angiogenic signals are generated from VEGFR-2, whereas sFlt-1 maintains physiological corneal avascularity and induces several symptoms in preeclampsia. VEGFR-1 is expressed not only in endothelial cells but also in monocytes and macrophages, and it stimulates angiogenesis, inflammation, and the malignant phenotypes of cancer. Nrp: co-receptors for VEGFs such as $VEGF\text{-}A_{165}$, which increase the affinity between VEGF and its receptors, and the intracellular signaling; VEGFxxxb: an alternatively processed variant form of VEGF-A that is suggested to be an endogenous competitor against VEGF-A. *Source: Reprinted with permission from Ref. 280.*

binds to both receptors, as well as a co-receptor neuropilin 1; VEGF-B only binds to the Flt-1 receptor, which also binds PlGF. VEGF-C and -D bind to another member of this receptor family, fms-like tyrosine kinase 4 (Flt-4, or type 3), and stimulate lymph angiogenesis, while VEGF-E is a KDR–Flk-1 agonist. Results from Flt-1 tyrosine kinase-deficient mice indicate that Flt-1 signaling is important for tumor growth, metastasis, and chronic inflammation (Figure 40.7).[280,284] Upon VEGF or FGF2 activation, the receptor tyrosine kinases initiate a cascade of cellular protein phosphorylations by protein kinases, notably ERK1/2, Akt-1, and/or p38 MAPK, resulting in enhanced proliferation and migration of fetal placental artery endothelial cells.[285] The VEGF-induced signaling mechanisms are highly complex, and an endothelial cell-specific chemotaxis receptor, which is expressed in the placenta, co-localizes with and is required for activation and internalization of the KDR–Flk-1 receptor in endothelial cells.[286] A soluble truncated form of the Flt-1 receptor (sFlt-1), which is generated as a splice variant and contains an extracellular binding domain but lacks tyrosine kinase signaling, has tremendous capacity to sequester VEGF as well as PlGF and thus prevent VEGF interaction with the Flt-1 and KDR–Flk-1 receptors.[287,288]

The mRNAs and proteins for VEGF, Flt-1, KDR–Flk-1, PlGF, and the related angiopoietin 1 and 2 and Tie2 receptor molecules are abundantly expressed by cytotrophoblasts, the syncytiotrophoblast, and/or inner villous stromal cells within the placenta, as well as EVTs, interstitial, and/or decidual cells within the placental basal plate during human,[289–291] baboon,[292] rhesus and marmoset monkey,[293–295] and ovine[296,297] pregnancy. VEGF mRNA expression in villous cytotrophoblasts and vessel density within the inner villous compartment increase with advancing human[298–300] and baboon[292] gestation. As detailed in the section Estrogen, the increase in placental E_2 production with advancing baboon pregnancy stimulates villous VEGF expression and establishment of the villous vasculature.[301] VEGF-A and the Flt-1 and KDR–Flk-1 receptors also appear to be critical for trophoblast function in the extravillous placenta, since the migration and invasive capacity of EVTs were suppressed by blocking VEGF action in cultures of human placental trophoblasts.[302,303] Also as discussed in the section Estrogen in contrast to the E_2-induced increase in VEGF expression by cytotrophoblasts in the placental villous compartment, E_2 decreased VEGF expression by EVTs in the placental extravillous compartment and trophoblast remodeling of the uterine arteries in the decidua basalis during early baboon pregnancy.[304] The authors proposed, therefore, that the increase in E_2 of advancing primate pregnancy controls the extent to which the uterine arteries are remodeled, while concomitantly promoting vascularization of the villous placenta, thereby promoting maternal blood flow to the placenta and thus delivery of substrate to the fetus to stimulate growth. Although the mechanisms of E_2 action on placental VEGF expression remain to be determined, in the rat uterus E_2 enhances VEGF mRNA expression via activation of PI3K and Akt, which facilitates co-recruitment of estrogen receptor alpha (ERα) and HIF-1α to the promoter of the VEGF gene (Figure 40.8).[305]

Several studies have shown that placental expression and serum levels of VEGF and PIGF are lower and the sFlt-1 receptor that sequesters VEGF is higher in human pregnancies complicated by preeclampsia and FGR.[288,306–308] Because the Flt-1 gene promoter contains a hypoxia response element (HRE), the increase in placental hypoxia induced by subnormal uterine artery remodeling in women with preeclampsia may account for the increase in placental sFlt-1 expression,[309,310] and among other consequences also disrupt the permeability of renal glomerular endothelial cells, resulting in proteinuria,[311] a major symptom of preeclampsia. Moreover, Torry and colleagues[312] showed that nitric oxide (NO) generation prevented the rise in sFlt-1, but not VEGF, expression induced by hypoxia in human placental trophoblast cultures, indicating that NO had divergent effects on pro- and anti-angiogenic factors.

In cultures of placental BeWo cells, VEGF activated nuclear factor erythroid 2-related factor-2 (Nrf2), which enhanced transcription of those antioxidant response

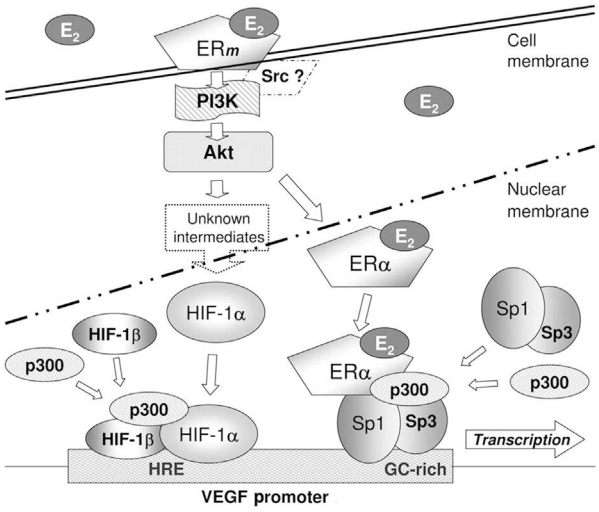

FIGURE 40.8 Model illustrating steps in estradiol (E_2) induction of VEGF expression in the rat uterus in vivo. Previous chromatin immunoprecipitation results indicate that estrogen simultaneously induces the recruitment of both HIF-1 (α and β) to the upstream HRE and ERα to the proximal GC-rich region of the VEGF promoter, probably via interaction with Sp proteins; p300 binds to both transcription factor complexes. The current results show that the activation and recruitment of both HIF-1α and ERα are mediated by PI3K. This may involve activation of a membrane form of the ER (here designated ER*m*), possibly through c-Src. *Source: Reprinted with permission from Ref. 305.*

element safeguard genes (e.g., glutathione peroxidase and heme-oxygenase 1 (HO1)) that are decreased in placentas of women with preeclampsia.[313] The increase in HO1 induced by VEGF also augmented the production of carbon monoxide and HIF-1α, which in a positive feedforward manner further elevated VEGF expression. The authors[313] suggested that a decrease in VEGF bioavailability during preeclampsia results in a decrease in Nrf2 and thus enhanced vulnerability to placental oxidative stress cell damage as well as a further reduction in VEGF.

PlGF has a 42% amino acid sequence identity with VEGFA and is expressed in four isoforms, PlGF1–4, as a result of alternative splicing. PlGF binds specifically to the Flt-1 receptor, and in addition to promoting EVT migration and invasive capacity,[306,307] it has been reported to have both pro- and anti-angiogenic effects.[314] In retinal microvessel endothelial cells, PlGF1 stabilized the VE-cadherin and claudin 5 components of adherens and tight junctions, thereby inhibiting VEGF-induced endothelial cell permeability, a critical early step in angiogenesis.[315]

Endocrine Gland-VEGF

In 2001, Ferrara and colleagues[316] identified a new growth factor that was expressed only in steroid-producing EGs (i.e., the ovary, testis, adrenal, and placenta). Because this protein was mitogenic for EG endothelial cells and exhibited other features characteristic of VEGF (e.g., induction of fenestrations in target cells and upregulation of the gene promoter by hypoxia), it was named EG-VEGF. However, EG-VEGF shares no structural homology with VEGF, but rather is structurally related to a family of peptides termed prokineticins that includes Bv8 protein, also named prokineticin 2 (PROK2).[317,318] The mature EG-VEGF protein is composed of 86 amino acids, including 10 cysteines, and is structurally identical to prokineticin 1 (PROK1). The gene for EG-VEGF–PROK1 is located on chromosome 1p21 and composed of three exons with no known alternative splicing products. Two G-coupled receptors (PROKR1 and PROKR2) bind PROK1 and PROK2 with equally high affinity and mediate a cellular response dictated by the repertoire of G proteins. Activation of the downstream PROK signaling pathways involves MAPK–Akt phosphorylation, phosphoinositol turnover, and mobilization of cAMP or calcium.[318]

EG-VEGF mRNA and protein are detected in the human placenta throughout the course of gestation, and they are localized abundantly in the syncytiotrophoblast and lightly in the cytotrophoblast, but not in the EVTs.[319] Placental expression and maternal serum levels of EG-VEGF peak at 8–9 weeks of gestation and decline thereafter, a pattern very similar to that of hCG. The PROKR1 and 2 receptors are expressed throughout gestation and localized to the syncytiotrophoblast, villous cytotrophoblast, and EVTs.[319,320]

Although expression of EG-VEGF and its receptors can be upregulated by hypoxia, recent studies indicate that hCG may play a role as well.[321] Thus, using placental explants and primary cultures of trophoblast from the first trimester of human pregnancy, Brouillet et al.[321] showed that the cytotrophoblast and syncytiotrophoblast expressed the LH–hCG receptor and that hCG increased mRNA and protein expression of EG-VEGF and PROKR2 in the syncytiotrophoblast and PROKR1 in the cytotrophoblast in a time- and dose-dependent manner. These investigators also cloned the promoter of the EG-VEGF gene and documented the existence of two canonical cAMP response elements (CRE1 and CRE2). Moreover, gene reporter assays with COS7 cells (African green monkey kidney cells) that transiently expressed the luciferase gene under control of the EG-VEGF promoter showed that cAMP rapidly enhanced promoter activity. Thus, cAMP-induced expression of the EG-VEGF promoter occurs at the level of transcription and is mediated via activation of CRE2. Based on these findings, Brouillet et al.[321] have proposed that hCG regulates placental development via control of expression of EG-VEGF and its receptors (Figure 40.9).

It has also been suggested that EG-VEGF may play a role in EVT invasion based on its pattern of secretion and regulation by hypoxia, the marked expression of PROKR2 receptor in EVTs, and the demonstration that maternal serum levels and placental expression of EG-VEGF are elevated in women with preeclampsia.[318] In support of this suggestion, EG-VEGF suppressed the invasive capacity of trophoblasts prepared from normal placentas obtained at 7–12 weeks of gestation or transformed EVT cells.[320] However, it remains to be determined whether EG-VEGF plays

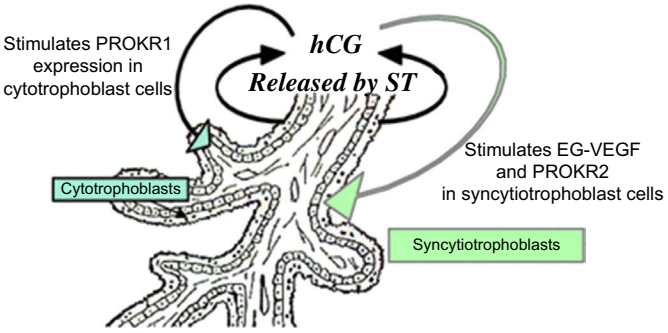

FIGURE 40.9 Proposed model of hCG stimulation of the EG-VEGF–PROKR1 and PROKR2 system in the human placenta during the first trimester of pregnancy. hCG released from the syncytiotrophoblast layer will activate EG-VEGF and PROKR2 expression in the ST layer in an autocrine manner and PROKR1 expression in the cytotrophoblast layer in a paracrine manner. *Source: Reprinted with permission from Ref. 321.*

a physiologic role in regulating EVT invasion or becomes important later in gestation in exacerbating the placental dysfunction characteristic of pregnancy pathologies (e.g., preeclampsia).

It is evident, therefore, that each of the PlGFs (i.e., IGF-I/-II, TGFβ, EGF, VEGF, and EG-VEGF) has been shown by in vitro studies to have the capacity to control placental villous and EVT function, particularly EVT migration and invasive capacity. Future investigation is needed in this area of placental biology to establish the applicability of the role of the PlGFs in vivo during human pregnancy and the manner in which these factors are regulated and interact to regulate placental function.

STEROID HORMONES OF THE PLACENTA

Detailed reviews of the historical perspectives, biosynthetic steps, and characterization of the enzymes involved in the production of placental estrogens and P_4 have previously appeared.[322–326] The present section provides an overview of synthesis and recent developments in the regulation and actions of the placental steroid hormones.

Estrogens

Synthesis

The placenta becomes the primary source of the estrogens at approximately 8 weeks of human gestation, and serum estrone, E_2, estriol, and estetrol levels progressively increase thereafter (Figure 40.3), with total daily production rates of these estrogens at term approximating 40 mg. The placenta does not express the 17α-hydroxylase–17-20-lyase ($P450_{C17}$) gene required for de novo production of the estrogens. However, the fetal zone of the fetal adrenal cortex is a major source of C_{19} steroids, notably dehydroepiandrosterone (DHEA), that are aromatized via the P450 aromatase (P450arom) to the estrogens.[327,328] Because of the importance of the fetal adrenal for placental estrogen synthesis, disorders (e.g., anencephaly) or administration of synthetic corticosteroids that suppress fetal pituitary–adrenocortical function result in a 50% decline in estrone and E_2 and a 90% decrease in estriol levels.[329,330] The maternal adrenal provides the C_{19} steroids for the remaining 50% of estrone and E_2 formation during pregnancy. The concept of a functional fetal–placental unit for estrogen biosynthesis in human pregnancy provided the basis in the past for measurement of maternal serum estriol levels to assess fetal well-being and placental uptake of DHEA to evaluate placental function.

Within the fetal adrenal cortex, DHEA is synthesized de novo from cholesterol, derived primarily by receptor-mediated uptake of LDL, and then sulfurylated and delivered to the placenta as DHEAS or, after 16-hydroxylation in the fetal liver, as 16-0H-DHEAS (Figure 40.10). DHEAS is converted to DHEA and 16-OH-DHEAS to 16 OH-DHEA via a sulfatase expressed in the placenta. DHEA is converted to Δ^4-androstenedione via Δ^5-3β-hydroxysteroid dehydrogenase–isomerase (3β-HSD), and a single P450arom enzyme; anchored to the endoplasmic reticulum and encoded by the CYP19A1 gene located on chromosome 15q 21.1; aromatizes Δ^4-androstenedione to estrone, testosterone to E_2, and 16-OH-DHEA to estriol. The CYP19A1 gene contains five different transcriptional start sites and individual promoters for tissue-specific expression in the placenta and other tissues. The placenta utilizes exon 1.1 for the transcription start site that binds the Sp1 transcription factor.[331] P450arom catalyzes the oxidative demethylation of C_{19} steroids to yield C_{18} steroids with an aromatic A-ring. The crystal structure of P450arom has been characterized and in the crystal aromatase molecules are uniquely linked by head-to-tail intermolecular interaction via a loop between helix D and helix E of one aromatase molecule that penetrates the heme-proximal cavity at the next molecule, forming in tandem a polymeric aromatase chain.[332,333] The interconversion of estrone and E_2 and of Δ^4-androstenedione and testosterone is catalyzed by 17β-hydroxysteroid oxidoreductase (17β-HSD).

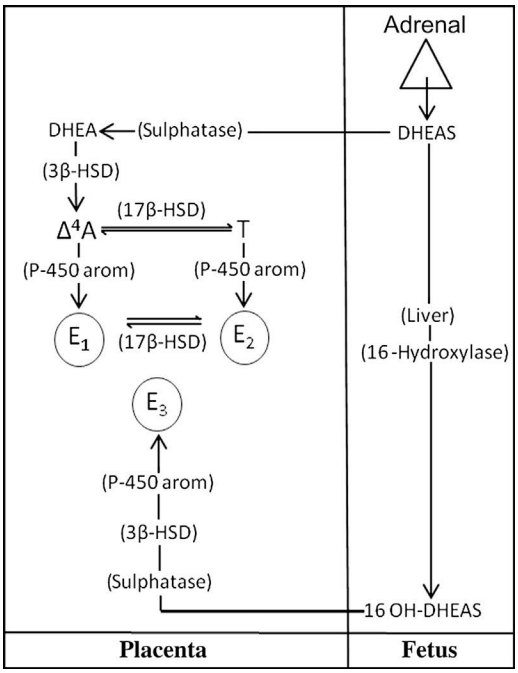

FIGURE 40.10 Estrogen biosynthesis in the fetal–placental unit during human pregnancy. DHEA, Dehydroepiandrosterone; DHEAS, DHEA sulfate; Δ^4A, Δ^4-androstenedione; T, testosterone; E_1, estrone; E_2, estradiol; E_3, estriol.

Several different 17β-HSD isoforms exist, which differ in structure, substrate specificity, and co-factor use. The interconversion of estrone and E_2 is catalyzed by 17β-HSD-1, located on chromosome 17q11–q21, and interconversion of testosterone and Δ^4-androstenedione is catalyzed by 17β-HSD-2, and both 17β-HSD-1 and -2 are expressed in the placenta.

Regulation

Since the fetal zone of the fetal adrenal cortex is a major source of DHEA and other C_{19}-steroid precursors that are aromatized to the estrogens in the placenta, the regulation of the fetal cortical zone is paramount to placental estrogen production. Therefore, as previously reviewed,[334–336] ACTH and other factors, including growth factors, that stimulate fetal adrenal DHEAS synthesis indirectly increase placental estrogen production. In addition to ACTH, corticotrophin-releasing hormone (CRH) secreted by the placenta has the capacity to stimulate fetal adrenal DHEAS production directly[337,338] and thus placental estrogen formation. Moreover, placental CRH and its related peptide urocortin 1 (Ucn1), acting via the CRH receptors (CRHR1 and CRHR2) that are expressed in the placenta[339] and that activated Gs–Gq PKA and Gi–Gq PKC signaling pathways,[340–342] enhanced E_2 and decreased P_4 formation by cultured human placental trophoblasts (Figure 40.11).[342–345] Since placental CRH and Ucn1 levels increase[346,347] and the ratio of plasma P_4 to estriol decreases[348] near term, it has been suggested that CRH and related peptides may be involved in the initiation of labor (see Chapter 42).

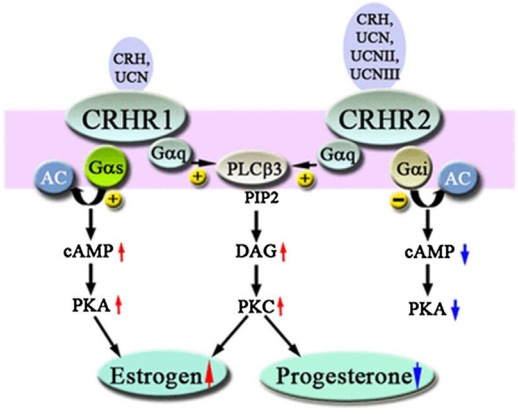

FIGURE 40.11 Scheme illustrating the proposed regulation of estrogen and progesterone production by CRH and its related peptides in human placenta. CRHR1 primarily couples to Gs and Gq protein. When CRH or UCN binds CRHR1, it stimulates the AC–cAMP–PKA and PLC–PCK signaling pathways, which stimulates estrogen production. CRHR1 activation can inhibit progesterone production via the PLC–PKC signaling pathway but not by the AC–cAMP–PKA signaling pathway. When CRHR2 is activated by UCNII and UCNIII as well as UCN and CRH, it induces Gαi and Gαq activation and then stimulates the PLC–PCK signaling pathway, which stimulates estrogen production and inhibits progesterone production. *Source: Reprinted with permission from Ref. 342.*

The capacity for placental aromatization increases with advancing gestation, and cAMP plays an important role in stimulating expression of the P450arom enzyme.[349–351] Cortisol has also been shown in human trophoblast cell cultures to increase the expression of P450arom by activating the cAMP–Sp1 pathway.[352] Moreover, in vitro differentiation of human cytotrophoblasts to the syncytiotrophoblast and the associated induction of P450 aromatase–CYP19A1 are upregulated in a 20% O_2 environment and prevented by 2% O_2, suggesting regulation by O_2-modulated transcription factors such as mammalian achaete-scute homologous protein 2 (Mash2)–achaete-scute complex homolog 2 (ASCL2).[353,354] In addition, the human placental trophoblast expresses the estrogen receptor alpha (ERα)[355] and an estrogen response element–like sequence that modulates the induction of CYP19 promoter 1.1 activity by E_2.[356] The human placenta also expresses the orphan nuclear estrogen-related receptor gamma (ERRγ),[357] which transgenic mouse studies suggest may regulate ion transport channels[358] and genes involved in angiogenic and calcium transfer pathways,[359] and which is selectively upregulated during trophoblast differentiation. ERRγ induces CYP19A1 and is downregulated by HIF-1α in placental cell culture.[360] Therefore, Mendelson and colleagues[360] have proposed (Figure 40.12) that early in gestation, when the placental environment is hypoxic, elevated levels of HIF-1α inhibit ERRγ transcription and thus CYP1A gene expression and cytotrophoblast differentiation into the syncytiotrophoblast. It is further proposed that after week 9 of gestation, the increase in placental vascularization increases O_2 availability and decreases HIF-1α, which causes upregulation of ERRγ and induction of CYP19A1 transcription. The resulting increase in E_2, in turn, is proposed to activate ERα, which may functionally interact with ERRγ and other transcription factors to further upregulate CYP19A1 expression.

Function

The placenta produces micromolar amounts of the estrogens during human pregnancy and, as presented in the accompanying Chapters 42 and 43 of this text and in earlier reviews, it is well established that E_2 has an important role in preparing the endometrium for implantation, the cascade of events leading to the induction of labor,[361] and the maternal cardiovascular changes of pregnancy.[362,363]

ER knockout mice have been used to characterize the role of E_2 in reproduction in rodents.[364] However, the human placental trophoblast also expresses ERα and ERβ,[355,365] providing a mechanism for E_2 action in

this organ during human pregnancy. For example, the morphological differentiation of human villous cytotrophoblasts into the syncytiotrophoblast is stimulated in vitro by E_2.[366,367] E_2 also stimulates trophoblast expression of leptin,[368–370] which along with other factors promotes trophoblast differentiation.[371] Using the baboon as a nonhuman primate model for the study of human pregnancy, the authors have shown that E_2 also controls several other important aspects of placental and fetal development. Remodeling of the uterine spiral arteries is extensive during the first trimester of human pregnancy, whereby EVTs migrate to, invade, replace the endothelial and smooth muscle lining of, and thus transform the uterine arteries into low-resistance high-capacity vessels to promote blood flow to the placenta and thus development of the fetus. Impaired vessel remodeling underlies the etiology of preeclampsia,[372] which results in maternal and neonatal morbidity and mortality, while excessive remodeling that occurs in placenta accreta impairs essential vasoregulatory processes after delivery.[373,374] Although there is a rapid rate of uterine artery transformation early in gestation when E_2 levels are low, prematurely elevating E_2 levels in early baboon pregnancy, by shifting the normal rise in E_2 from the second to the first trimester via maternal E_2 administration, suppressed trophoblast invasion and remodeling of the uterine spiral arteries[375] and disrupted uteroplacental blood flow dynamics at term.[376] The decline in vessel transformation was associated with a decrease in the expression of placental EVT VEGF and the α1β1 and α5β1 integrins thought to be involved in trophoblast migration.[304,377] Thus, VEGF and certain integrins may mediate the E_2-induced suppression of uterine vessel transformation. The lack of a direct effect of E_2 on HTR8-SV neo trophoblast migration and invasive capacity, as shown in vitro,[378] seems consistent with E_2 acting via other factors. The authors[379] have proposed, therefore, that the low levels of ovarian E_2 in early pregnancy promote VEGF expression and EVT remodeling of the uterine arteries (Figure 40.13), whereas the normal rise in E_2 thereafter suppresses and thus controls the extent to which the uterine vessels are remodeled. E_2 administration in early rat pregnancy also suppressed development of the uterine spiral arteries,[380] although the endocrine interrelationships between the mother, placenta, and fetus are different in rodents and in humans and nonhuman primates. The E_2-induced suppression of uterine artery transformation in early baboon pregnancy appears translationally relevant to the human, since elevated levels of E_2 in early in vitro fertilization human pregnancy are associated with a fivefold increased risk of developing preeclampsia,[381] in which subnormal trophoblast uterine artery remodeling is a hallmark feature. In the second trimester of baboon pregnancy, the normal rise in E_2 concomitantly promoted placental villous VEGF expression and blood vessel development,[301,382] as it does in myometrial microvascular endothelial cells.[383] Thus, E_2 differentially regulates placental EVT and villous VEGF expression and consequently coordinates uterine artery remodeling and villous angiogenesis to ensure blood flow to and vascularization of the villous placenta to promote fetal development.

During the second half of baboon pregnancy, E_2 stimulates the receptor-mediated uptake of LDL as cholesterol substrate for and activity of the P450scc enzyme catalyzing the synthesis of P_4 by the placental trophoblast.[384–386]

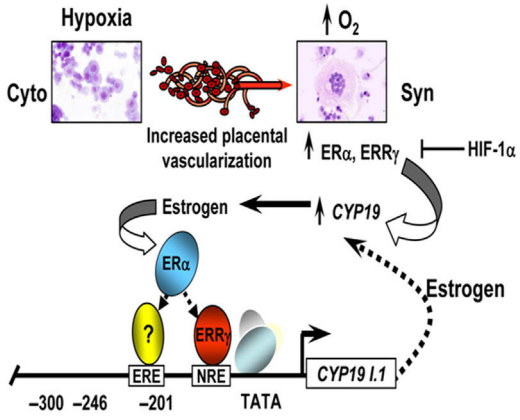

FIGURE 40.12 Model of hCYP19 regulation in human trophoblasts. This figure is reproduced in color in the color plate section. Early in gestation, the placenta is relatively hypoxic; the elevated levels of HIF-1α inhibit ERRγ transcription via interaction with a response element (HRE) in the ERRγ promoter. This, together with hypoxia-mediated induction of basic helix–loop–helix factors ASCL2 and USF1/2, blocks syncytiotrophoblast differentiation and hCYP19 gene expression. After the ninth week of gestation, increased placental vascularization results in increased oxygen availability to the trophoblast cells. The consequent decrease in HIF-1α levels and the expression of other inhibitory transcription factors cause upregulation of ERRγ and induction of hCYP191.1 transcription. The increased estrogens produced, in turn, activate ERα, which may functionally interact with ERRγ and other activating transcription factors to further upregulate hCYP19 expression. *Source: Reprinted with permission from Ref. 360.*

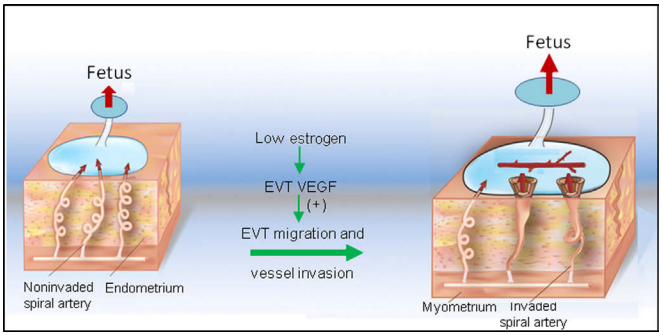

FIGURE 40.13 Proposed role of low estrogen levels in early primate pregnancy in promoting VEGF expression and extravillous trophoblast (EVT) migration and invasion–remodeling of the uterine spiral arteries.

In addition to regulating syncytiotrophoblast expression of the 11β-hydroxysteroid dehydrogenase 1 and 2 (HSD1/2) enzymes responsible for controlling transplacental cortisol–cortisone metabolism, as discussed in the section 11β-Hydroxysteroid Dehydrogenase (11β-HSD) and Cortisol–Cortisone Metabolism, the increase in E_2 of advancing baboon gestation downregulates cyclin D1 expression within[387,388] and thus the growth of and production of C_{19} steroid estrogen precursors by the fetal zone of the fetal adrenal cortex, apparently in a feedback manner to control E_2 levels within a physiological range.[389] As highlighted by Kaludjerovic and Ward,[390] the E_2-induced alteration of fetal adrenocortical maturation may translate to the human and be elicited by exposure to environmental estrogens as mediated by epigenetic changes.

Wood and colleagues[391,392] have shown that E_2 increases activity of, and ACTH secretion within, the fetal hypothalamic–pituitary axis during ovine pregnancy, possibly via modulating fetal brain prostaglandin biosynthesis.[393] Thus, Wood has proposed that the interplay of placental E_2 and the fetal hypothalamic–pituitary–adrenal (HPA) axis may have a role in the initiation of labor in the human.[392]

Development of the pool of ovarian follicles essential for reproductive function in adulthood in the human is established in utero and in the baboon fetus, in which the levels of E_2 were suppressed throughout the second half of pregnancy by maternal administration of the aromatase inhibitor letzozole, the number of ovarian follicles was reduced by 50%,[394] and the majority of the remaining follicles contained oocytes that exhibited both vacuolization and disrupted mitochondria.[395] The disruption of fetal ovarian follicle formation by E_2 suppression was not due to subnormal serum FSH levels or ovarian FSH receptor expression.[396] However, the number and height of oocyte microvilli were decreased,[395] the subcellular localization of the ezrin and ezrin-binding protein 50 proteins critical for microvillus formation were altered,[397] and the intraovarian activin–inhibin ratio was decreased[398,399] by E_2 deprivation. Each of these changes, along with ovarian follicle formation, was restored by concomitant E_2 and letrozole treatment. The authors[379,400] have proposed, therefore, that E_2 promotes formation of the stockpile of healthy ovarian follicles within the primate fetus (Figure 40.14) and that this developmental process is critical for the timely onset of puberty and reproductive function after birth.[401] The human fetal ovary also contains the mechanisms required to respond to E_2, and Fowler and colleagues[402] have proposed that E_2 also plays a role in primary follicle formation in the human fetus, a postulate that is consistent with the abnormal ovarian follicle development that occurs in women with P450arom deficiency.[403]

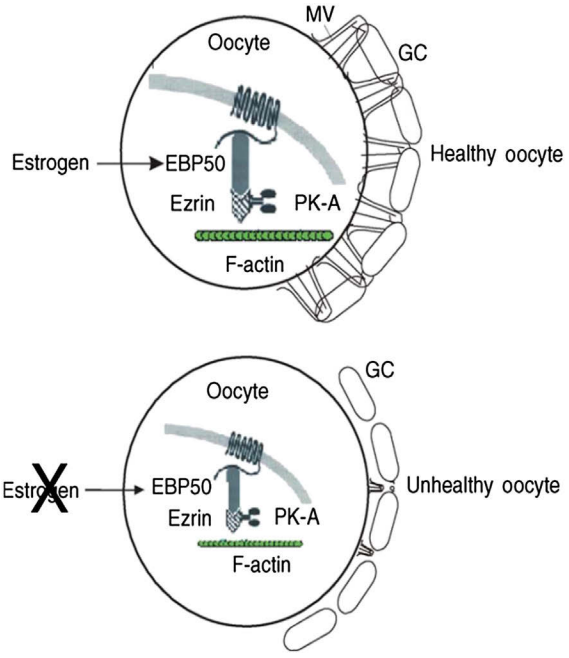

FIGURE 40.14 Proposed role of estrogen on fetal ovarian oocyte microvillus (MV) formation and interaction with granulosa cells (GCs) essential for oocyte health and function and thus long-term survival. During primate pregnancy, estrogen promotes the expression and subcellular location of the ezrin and ezrin binding protein 50 (EBP50) proteins that are critical for attaching to F-actin and thus microvillus formation. *Source: Reprinted with permission from Ref. 400.*

E_2-deficient pregnant baboons,[404] P450arom or ERα knockout mice,[405–408] and P450arom-null humans[409,410] exhibit a disruption in testis morphology; germ cell, efferent ductule, and epididymal development; and/or infertility, showing that E_2 also has an essential role in promoting fetal testis development and fertility in adulthood.

Null mutation of P450arom in human pregnancy[411–413] and administration of the aromatase inhibitor letrozole during baboon pregnancy[414] also result in insulin insensitivity and glucose intolerance in the offspring, indicating that E_2 in utero programs mechanisms within the fetus that are important for normal insulin action after birth. Placental E_2 deficiency in human pregnancy also impairs the pubertal growth spurt, skeletal maturation, and accrual of bone mass in both men and women.[403,415,416]

Progesterone

Synthesis

The placenta also becomes the principal source of P_4 at approximately 8 weeks of human pregnancy, and P_4 levels increase to 150–175 ng/ml at term (Figure 40.3) and a production rate of over 200 mg/day. De novo synthesis of cholesterol from acetate and cholesterol

esterification within the placenta are limited, whereas the receptor-mediated uptake, utilization, and metabolism of LDL by the placental syncytiotrophoblast via the classical LDL pathway account for the major proportion of placental P_4 production.[417] The apolipoprotein component of LDL and cholesteryl esters are then hydrolyzed to form free cholesterol. Placental mitochondrial transport and binding of cholesterol are not mediated via steroidogenic acute regulatory protein (StAR), which is not expressed in the placenta. However, MLN64 protein, which is structurally closely related to StAR,[418] has the ability to transport cholesterol between membranes of and stimulate pregnenolone synthesis by isolated placental mitochondria.[419] Cholesterol is converted to pregnenolone via the P450 cholesterol side-chain cleavage (P450scc) enzyme attached to the matrix side of the inner mitochondrial membrane. This process involves hydroxylation and oxidative scission of the C20-22 bond of 20,22-dihydroxycholesterol. A single P450scc protein, termed CYP11A1, lies on chromosome 15q23–q24 and is composed of nine exons that span 30 kb. Unlike in the adrenal and gonad, expression of P450scc in the placenta does not require steroidogenic factor 1 (SF1), but CP2 proteins[420,421] and zinc finger protein TReP-132[422] mediate placental P450scc expression. P450scc functions as a terminal oxidase in a mitochondrial electron transport system, whereby electrons from NADPH are accepted by an adrenodoxin reductase flavoprotein associated with the inner mitochondrial membrane. Adrenodoxin reductase transfers the electrons to an iron–sulfur protein, adrenodoxin, which is located in the mitochondrial matrix and acts as an electron shuttle to donate the electrons to cytochrome P450scc. Tuckey et al.[419,423,424] have shown that the reduction of adrenodoxin by adrenodoxin reductase is a rate-limiting step for electron transport to P450scc in the placenta. A 3β-HSD enzyme catalyzes the conversion of pregnenolone to P_4 and DHEA to Δ^4-androstenedione. Placental 3β-HSD type 1 is located on chromosome 1p13.1 and is a microsomal monomer peptide of 42,000 Da located in both mitochondrial and microsomal fractions. Dehydrogenase and isomerase activities of 3β-HSD are inseparable and catalyze both C_{19} and C_{21} Δ^5 to Δ^4 steroid reactions using NAD^+ as cofactor. It appears that placental 3β-HSD is in excess and thus not rate limiting for P_4 synthesis.

Regulation

Placental P_4 biosynthesis depends on the LDL receptor-mediated uptake of substrate LDL; however, placental P_4 synthesis does not appear to be acutely regulated. cAMP chronically stimulates expression and activity of P450scc,[425,426] adrenodoxin,[425] LDL receptors,[427] and P_4 secretion[428] in a dose-dependent manner in cultures of human cytotrophoblasts, an effect that requires the catalytic unit of protein kinases, which may regulate gene transcription.[425] PKA and phosphotyrosine phosphatase D1 are associated with the syncytiotrophoblast mitochondrial membrane by an anchoring kinase cAMP protein-121, and PKA inhibitor H89 suppressed P_4 synthesis.[429] As discussed here, the reduction of adrenodoxin is a rate-limiting step for electron transport to P450scc, and cAMP has been suggested to have a role in regulating placental adrenodoxin.[324] Placental mitochondrial P450scc activity in humans[417] and P450scc mRNA activity in baboons[430] increase with advancing gestation, and, as discussed here, E_2 stimulated the LDL uptake and P450scc activity steps involved in placental P_4 formation during baboon pregnancy.

Function

As presented in Chapter 38 in this book on implantation, P_4 has an essential role in promoting endometrial decidualization, implantation, and glandular function in the early establishment of pregnancy. Transgenic mouse studies show that P_4, signaling through progesterone receptor (PR) isoforms A and B as modulated by COUP (chicken ovalbumin upstream promoter) transcription factor 2 nuclear receptor and Heart- and neural crest derivatives expressed protein 2 transcription factor, is critical for decidualization and implantation.[431] P_4 also suppresses the maternal immune system and inflammatory processes at the maternal–fetal interface to promote pregnancy maintenance and implantation of the conceptus,[432,433] as presented in Chapter 41. P_4 also suppressed the migration and invasive capacity of HTR8–SV neo trophoblast cells in vitro[378] by inhibiting MMP2 and MMP9 activities,[378,434] and thus has the potential to control EVT vessel invasion in early pregnancy. As detailed in Chapter 42 on parturition, P_4 has a pivotal role throughout pregnancy in suppressing myometrial contractility and labor. Finally, transgenic mouse studies indicate that PR action is essential for lobuloalveolar proliferation and growth of the mammary gland,[431] but suppresses secretory activity of the breast until after delivery when placental P_4 is lost.[435]

Physiological Relevance and Clinical Correlations

The results of P450arom deficiency in human pregnancy, administration of aromatase inhibitor during nonhuman primate pregnancy, and developmental and in vitro studies demonstrate the fundamentally important role that placental E_2 plays during human and nonhuman primate pregnancy. These studies collectively have shown that E_2 controls implantation, placental EVT and villous development, transplacental corticosteroid metabolism, fetal adrenal and gonadal development, metabolic and cardiovascular dynamics, and the initiation of labor. Although the incidence of P450arom deletion in human pregnancy seems rare, it is possible that cases of aromatase deficiency may have been overlooked

because of the need for E_2 during implantation and early placental development and the lethality in its absence. Because pregnancy and fetal development appeared normal in cases of human placental sulfatase[436] and aromatase[403,416,437] deficiency, it has been concluded[326,403] that estrogen is not needed for normal fetal development and the physiology of pregnancy. However, the difficulty of conducting comprehensive investigation of physiological and developmental processes and mechanisms during human pregnancy limits the conclusions that can be drawn from the clinical studies. Most importantly, detailed follow-up studies of the offspring delivered from estrogen-deprived human and nonhuman primate pregnancy are needed to fully establish the importance of E_2 in utero on fetal organ system development and the impact on physiological processes and metabolic function after birth.

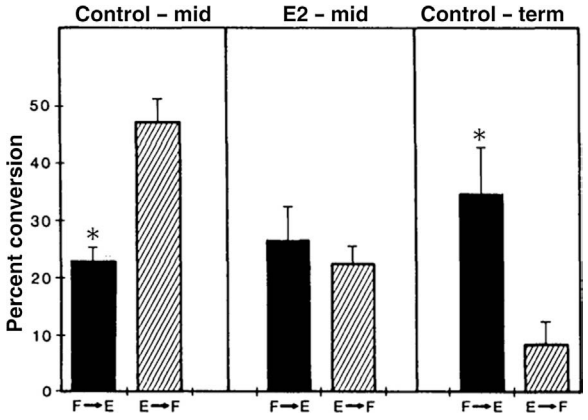

FIGURE 40.15 Transuteroplacental conversion of cortisol (F) to cortisone (E) and of E to F in untreated control baboons on gestation days 100 (midgestation) and 170 (term) and in baboons treated daily with E_2 between days 70 and 100 (midgestation). Values (mean ± standard error) represent metabolism by the uterus and placenta corrected for fetal contributions. * Indicates that percentage of F→E differs from percentage of E→F at $P < 0.05$. *Source: Reprinted with permission from Ref. 440.*

11β-Hydroxysteroid Dehydrogenase (11β-HSD) and Cortisol–Cortisone Metabolism

Developmental Pattern of Cortisol–Cortisone Metabolism in the Primate Placenta

It has long been known that the placenta in late gestation in the human[438] and nonhuman primate[335] actively metabolizes cortisol to cortisone. Because cortisol binds to the glucocorticoid receptor, whereas cortisone does not and is inactive, it has generally been thought that the major if not sole role of the primate placenta is to protect the fetus from the relatively high concentrations of biologically active cortisol present in maternal circulation.[335] While the latter is indeed a major function of the placenta, as discussed in more detail in this chapter, Pepe and Albrecht[439,440] showed that the primate placenta also exhibits the capacity to form cortisol from cortisone. Moreover, during early to midgestation, placental formation of cortisol from cortisone is substantial and exceeds the oxidation of cortisol to cortisone. Thus, the pattern of placental glucocorticoid metabolism is altered from preferential reduction of cortisone to cortisol at early to midgestation, a time in pregnancy when the fetus and fetal adrenal cannot synthesize their own cortisol, to preferential oxidation in late gestation (Figure 40.15). It has been proposed, therefore, that the placenta by controlling the formation of cortisol from cortisone dictates the levels of cortisol that arrive in the fetal circulation, consistent with the prevailing theory of the importance of the maternal–placental hormonal milieu in programming fetal organ system development.[335]

It has also been shown that E_2 regulates placental cortisol–cortisone metabolism.[440,441] Thus, the developmental change in placental corticosteroid metabolism from preferential reduction of cortisone to cortisol at midgestation to oxidation of cortisol to cortisone at term was prevented in baboons in which E_2 production was suppressed and induced prematurely at midgestation by E_2 administration (Figure 40.15). In contrast, the oxidation–reduction of cortisol–cortisone in maternal tissues was not altered during the course of pregnancy or by E_2.[442] Thus, estrogen specifically modulates metabolism in the syncytiotrophoblast as confirmed by studies showing that the conversion of cortisol to cortisone by cultures of baboon syncytiotrophoblast obtained at term exceeds that of cells of midgestation and is increased in trophoblast cells from mothers treated at midgestation with E_2. It would appear that E_2 regulates placental glucocorticoid metabolism via interaction with ER expressed in the primate trophoblast.[442]

Regulation of 11β-HSD-1 and -2 Expression in Human and Baboon Placental Syncytiotrophoblast by E_2

In tissues such as the liver, cortisol metabolism is catalyzed by an 11β-HSD enzyme that exhibits both oxidative and reductive capacity and utilizes $NADP^+$–NADPH as co-factor. Although $NADP^+$-dependent 11β-HSD enzyme activity is demonstrable in the baboon[443] and human[444] placenta, the authors[439] were the first to show that 11β-HSD-catalyzed oxidation of cortisol to cortisone in homogenates of human and baboon villous trophoblast was much greater when NAD^+ was used as co-factor. Subsequent studies confirmed that NAD^+ is the preferred co-factor for the 11β-HSD enzyme catalyzing cortisol oxidation in the human placenta.[445] Collectively, these findings were consistent with the original suggestion of Monder and Shackleton[446] that there are multiple (iso) forms of 11β-HSD. Indeed, it has since been confirmed that there are two 11β-HSD

enzymes catalyzing cortisol–cortisone metabolism[447,448] and that these proteins are the product of two different genes.[449,450] The enzyme originally purified from rat liver[451] and termed 11β-HSD-1 is an $NADP^+$–NADPH-dependent oxido-reductase that behaves primarily as a reductase in part due to its higher affinity for cortisone. In contrast, 11β-HSD-2, which has been purified from human placenta,[452] is an NAD^+-dependent enzyme that only oxidizes cortisol to cortisone.

The 5′-flanking regions of the human and baboon genes encoding the 11β-HSD proteins[449,453–455] are >95% homologous, and the syncytiotrophoblast is a predominant site of expression of the mRNA and protein for both 11β-HSD-1 and -2.[456,457] Because 11β-HSD-1 can function as an NADPH-dependent reductase, the expression of 11β-HSD-1 in syncytiotrophoblast provides the molecular basis for the extensive reduction of cortisone to cortisol across the primate placenta during early to midgestation.[443] The syncytiotrophoblast expression of 11β-HSD-2 is consistent with the extensive capacity of the placenta to oxidize cortisol to cortisone.[335]

Studies have shown that the mRNA and protein levels of 11β-HSD-2, as well as 11β-HSD-1, in the syncytiotrophoblast increase with advancing gestation in the baboon and human.[456,458] Moreover, syncytiotrophoblast microsomal NAD^+-dependent 11β-HSD enzyme activity at midgestation was increased fourfold in baboons treated with E_2 and decreased at term in estrogen-suppressed animals.[442] The upregulation of 11β-HSD-2, and also 11β-HSD-1, in the syncytiotrophoblast appears to involve a direct ER-dependent action of E_2 on the promoter of these two genes.[454] Thus, in JEG-3 cells transfected with the 5′-flanking regions of the baboon 11β-HSD-1 or 11β-HSD-2 genes, basal promoter activities of both genes were increased by E_2 in the presence but not absence of a co-transfected ER. Consistent with these observations, Burton et al.[459] demonstrated that the mRNA and protein levels of 11β-HSD-1 and -2 in rat uterus were abolished by ovariectomy and restored by E_2 replacement.

The E_2-dependent upregulation of 11β-HSD-2 is consistent with increased transplacental oxidation of cortisol to cortisone at term.[443] However, the concomitant upregulation of 11β-HSD-1 mRNA, protein, and enzymatic activity with advancing gestation is surprising and presumably reflects a role for factors and/or processes in addition to upregulation of enzyme expression. One of the unique features of the syncytiotrophoblast is that it is a polarized cell composed of a maternal-facing microvillus brush border and a basal membrane (BM) juxtaposed to the fetal vasculature. Moreover, cells of other tissues in which 11β-HSD-2 is expressed (e.g., the colon and cortical collecting duct of the kidney)[457] are also polarized with different enzymes located in the luminal and BMs. The same appears to be the case for 11β-HSD-1 and -2 in the human and baboon placenta.[460] Thus, 11β HSD-1 is localized primarily in the syncytiotrophoblast microvillus membranes (MVMs) and is present, but in much lower amounts, in more internal regions, including the BM. In contrast, 11β-HSD-2 is localized to the cytosol and BM but not present in the MVM. Most interestingly, the ratio of 11β-HSD-2 to 11β-HSD-1 proteins in the BM is increased twofold between mid- and late gestation, an increase that is regulated by E_2.[461] It would appear therefore that the differential localization and increased levels of these two enzymes provide the biochemical–architectural basis for the E_2-dependent change in placental metabolism of cortisol–cortisone with advancing gestation. Consistent with this hypothesis, Burton et al.[462] have shown that 11β-HSD-1 and -2 expression is markedly different in the basal and labyrinth zones of the rat placenta. Finally, while E_2 has a pivotal role in establishing the extent and site of expression of these two enzymes, other factors apparently then function at discrete periods of gestation to fine-tune enzyme expression. Thus, human placental 11β-HSD-2 expression has been shown by in vitro studies to be upregulated by glucocorticoids, hCG, prostaglandins, and other factors that activate cAMP[463] and downregulated by hypoxia[464] and calcium.[465] Although study of the regulation of 11β-HSD-1 has been more limited, expression appears to be modulated by glucocorticoids and gene promoter activity downregulated in human epidermoid cancer 2 cells by growth hormone and IGF1.[466]

Impact of E_2-Regulated Placental Cortisol–Cortisone Metabolism on Fetal Development

In humans and nonhuman primates, the transitional zone of the fetal adrenal gland does not develop the 3β-HSD enzyme required to synthesize cortisol de novo until very late in gestation. Thus, throughout the majority of gestation, fetal adrenal cortisol production is negligible. In contrast, as judged by anatomical correlates of function, and the ability to secrete ACTH and CRH, the baboon and human fetal hypothalamus and pituitary gland develop the ability to function relatively early in gestation. Thus, development of the fetal adrenal appears to be "out of phase" with that of the hypothalamus and pituitary, yet by term the HPA axis has become functionally integrated.[442] Although cortisol is detectable in the fetus throughout the course of gestation, it is now well established that essentially all of the cortisol in fetal blood before midgestation is of maternal origin, whereas by late gestation >50% originates from hormone produced by the fetal adrenal.[443] Therefore, the authors[467] proposed that the placenta, via metabolism of cortisol–cortisone, dictates the qualitative and quantitative pattern of these corticosteroids arriving within the fetus, and consequently indirectly regulates the timing of maturation of the fetal HPA axis. As seen in Figure 40.16, because placental metabolism in early to midgestation

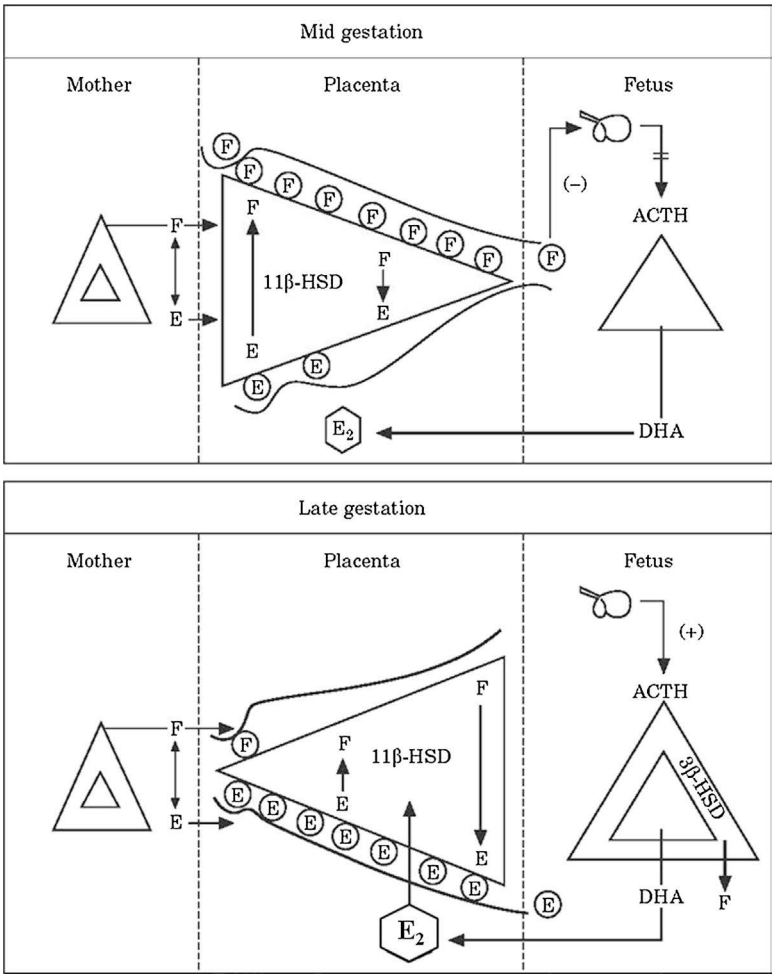

FIGURE 40.16 The role of estrogen (E_2) on placental 11β-HSD-catalyzed metabolism of cortisol (F) and cortisone (E) and regulation of the fetal pituitary–adrenocortical axis at mid- and late gestation in the baboon. During early to midgestation, E_2 levels are low and placental 11β-HSD-catalyzed metabolism of maternal glucocorticoids favors the formation and transfer of F to the fetus, which suppresses fetal pituitary ACTH production and development of the fetal adrenal transitional zone and thus F synthesis. With advancing gestation, the increase in E_2 enhances placental 11β-HSD-induced oxidation of maternal glucocorticoids to E, which decreases the amount of F arriving at the fetus; this results in increased fetal pituitary ACTH production, which induces 3β-HSD expression in and development of the fetal adrenal transitional zone and thus de novo F production by the fetus. 3β-HSD, Δ^5-3β-hydroxysteroid dehydrogenase. *Source: Reprinted with permission from Ref. 467.*

favors the formation of cortisol, the primary maternal corticosteroid arriving within the fetal circulation would be biologically active cortisol, which would suppress fetal pituitary ACTH production, thereby preventing maturation of the fetal adrenal transitional zone and the ontogenesis of cortisol production. With advancing gestation, the E_2-induced placental syncytiotrophoblast localization of 11β-HSD-1/-2 and increase in placental conversion of maternal cortisol to biologically inactive cortisone would be expected to decrease the concentration of maternal cortisol arriving across the placenta into the fetus, resulting in fetal pituitary ACTH release and maturation of the fetal adrenal gland. In support of this hypothesis, the expression of proopiomelanocortin mRNA and ACTH in the fetal pituitary,[468] mRNA–protein levels and activity of 3β-HSD and $P450c_{17}$, and protein kinase A activity in and the ontogenesis of de novo cortisol production by the baboon fetal adrenal were greater at term than at midgestation, and they increased prematurely at midgestation by treatment with E_2, which induced placental oxidation of cortisol to cortisone.[442] Subsequent studies in various rodent models confirm the earlier studies in the primate to support the important role of placental 11β-HSD enzymes in development of the fetal HPA axis.[469] For example, in mice, 11β-HSD-1 mutation leads to attenuated negative feedback of the HPA axis response to stress,[470] whereas mutation of the 11β-HSD-2 gene leads to hypertension, excess mineralocorticoid activity, and increased anxiety in adulthood.[471] Moreover, pharmacologic inhibition of 11β-HSD-2 during rodent pregnancy leads to molecular and neurological changes within the HPA axis associated with increased stress response and anxiety in adulthood.[472]

Physiological Relevance and Clinical Correlations

The placental 11β-HSD-dependent regulation of the fetal HPA axis is also likely operative during human pregnancy, as recently reviewed.[473] For example, consistent with the latter postulate, women with hypoadrenalism (e.g., Addison's disease) can maintain pregnancy without corticosteroid treatment,[474] ostensibly because of premature activation of the fetal HPA axis in utero in the absence of placental transport of maternal cortisol. Moreover, in low-birth-weight babies born at 27 weeks gestation, those with lower placental 11β-HSD-2 activity

had a more severe early postnatal clinical course and lower blood pressure and were more likely to receive glucocorticoid supplementation, consistent with decreased adrenal function.[475] Studies in humans have documented that placental[476] and fetal tissue[477] 11β-HSD-2 gene expression and activity are reduced in pregnancies complicated by FGR. However, placental expression of 11β-HSD-1, in addition to 11β-HSD-2, is reduced in babies born small for gestational age,[478] and umbilical cord blood levels of cortisol are low in FGR newborns.[479] Thus, while protection of the fetus from excess maternal cortisol, via placental 11β-HSD-2, appears to be an important factor in FGR, lower expression of 11β-HSD-1, which has not been investigated as extensively, might be equally important to compromised fetal growth and development. It is important to note that reduced expression of 11β-HSD-2 in FGR and growth restriction in pregnancies complicated by preeclampsia reflect measurements in late gestation. Interestingly, it has recently been shown that placental 11β-HSD-2 expression is actually elevated in the first trimester in pregnancies at risk for preeclampsia based on Doppler flow analyses.[458]

The importance of placental glucocorticoid metabolism to fetal well-being is further emphasized in animal experiments[480] and in human studies showing that stress experienced by the mother during pregnancy is associated with increased risk for adverse effects in the offspring, including anxiety,[481] attention deficit hyperactivity disorder,[482] and cognitive defects.[483] Moreover, placental 11β-HSD-2 expression was lower in mothers with maternal prenatal anxiety, presumably due to increased maternal adrenal cortisol production.[484] It has also been shown that cognitive and behavioral development was impaired in the offspring of mothers who consumed high levels of licorice, which contains glycyrrhetinic acid, an inhibitor of 11β-HSD-2.[485,486]

PLACENTAL HYPOTHALAMIC-LIKE NEUROPEPTIDE AND PEPTIDE HORMONES

It is well established that the placenta expresses key components of the hormonal systems that characterize the hypothalamic–anterior pituitary axis, including CRH–proopiomelanocortin (POMC)–ACTH, GnRH–LH–hCG, GHRH, and somatostatin, as well as the posterior pituitary (i.e., oxytocin). The placenta is also a source of several neuropeptides (e.g., kisspeptin, NPY, and neurokinin B (NKB)) and vasoactive peptides (e.g., endothelin 1, adrenomedullin (AM), calcitonin gene-related peptide (CGRP), and parathyroid hormone-related peptide (PTHrP)) that modulate other hormone systems that are important to successful pregnancy. The expression, regulation, and multiple actions and interactions of the placental hypothalamic-like hormones were reviewed by Petraglia and colleagues in the last edition of the *Physiology of Reproduction*.[255] In addition, reviews of the placental neuroendocrine system and roles in pregnancy and of pregnancies complicated by placental pathologies have also recently been published.[487–490] Thus, in this section, we present an overview of these hormones and recent advances on their regulation and roles in pregnancy and placental function.

CRH and Urocortins (Ucn)

The CRH–Ucn family of peptides is produced in the placenta and includes CRH, Ucn1, Ucn2, and Ucn3. The gene for CRH is located on chromosome 8 and contains a CRE, one-half of an estrogen response element, and two TATA boxes. The human Ucn gene is located on chromosome 2p23-p21, and putative regulators include a TATA box, GATA-binding sites, a POU domain, and a CRE. The free or biologically active levels of CRH and the Ucns are controlled primarily by a CRH-binding protein (CRHBP) also synthesized in the placenta. Importantly, in contrast to the marked increase in placental expression and maternal serum levels of CRH with advancing gestation, expression and levels of CRHBP decline sharply in the last 2–4 weeks of gestation,[491] presumably enhancing the bioavailability of CRH and Ucns.

CRH and Ucns exert their actions by activating one of two CRH receptors, CRHR1 (CRH and Ucn1) or CRHR2 (CRH, Ucn1, Ucn2, and Ucn3). Both receptors belong to the G protein-coupled receptor (GPCR) superfamily. The CRHR1 gene is located on chromosome 17q21.31 and expressed as eight splice variants, whereas the CRHR2 gene is located on chromosome 7q14.3 and transcribes three splice variants. The CRHRs are expressed in the syncytiotrophoblast, myometrium, amnion, and chorion.[492] CRH is expressed in the cytotrophoblast, syncytiotrophoblast, and intermediate trophoblast, while Ucn2 and Ucn3 are expressed by the syncytiotrophoblast, decidua, and EVTs.[493,494] The factors that control and thus increase (e.g., glucocorticoids, norepinephrine, and prostaglandins) or suppress (P_4) placental CRH production comparably influence hypothalamic CRH, with the exception of cortisol, which interestingly does not suppress but rather enhances placental CRH expression.[495] E_2 acting via ERα has been shown to upregulate CRHR gene expression in the syncytiotrophoblast, and activation requires a functional CRE.[496]

Placental CRH stimulates fetal pituitary ACTH expression[497] and via ACTH and/or a direct action increases fetal adrenal DHEA and DHEAS levels and thus placental estrogen synthesis, as well as cortisol production in late gestation.[337] As discussed in the section Steroid Hormones of the Placenta, CRH may also act directly

on the placental trophoblast to promote E_2 and decrease P_4 biosynthesis.[342] Because the rise in CRH after weeks 16–18 of gestation is more rapid in women who deliver preterm[498,499] and slower in those who deliver late, it has been proposed that CRH acts as a biologic clock to control the length of pregnancy and thus the timing of birth.[487,500–502] The mechanisms by which CRH and/or Ucn may control the timing of birth are unknown, although CRH and Ucn2 modulate myometrial contractility,[503] and the expression of the CRHR1 is upregulated in the human myometrium at the time of labor.[504] Ucn2 acting via CRHR2 regulates the PKC–MAPK–ERK signaling pathway and myosin light chain phosphorylation,[505] as well as large conductance calcium-activated potassium channels[506] in the human myometrium. Recent studies also indicate that the inducible nuclear transcription factor NFkB (or kB) also may play a role in modulating lipopolysaccharide- and TNFα-induced placental CRH transcription and labor.[507,508] Thus, TNFα, which is elevated by Ucn2,[509] activates interleukin 1 receptor-associated kinases, which complex with TGFβ-activated kinase ubiquitin-conjugating enzymes to activate an IkB kinase–NFkB essential modulator complex. The latter then phosphorylates an NFkB inhibitor releasing NFkB, which travels to the nucleus where it stimulates CRH transcription.

Glucocorticoids also increase the synthesis and nuclear translocation of the noncanonical NFkB pathway and consequently CRH expression in the human placenta.[508] Glucocorticoids[510] and CRH[511] also regulate placental trophoblast expression of the GLUT1 and GLUT3 transporters responsible for placental transfer of glucose to the fetus.[512] Additional investigation is needed to determine whether glucocorticoids act directly, or indirectly via placental CRH and placental transfer of substrates such as glucose, to disrupt programming of fetal growth and development. CRH–Ucn may also be involved in or interact with the pathways associated with preterm delivery due to infections,[513] as well as control EVT invasion and activation of Fas ligand-induced apoptosis in placentas of pregnancies complicated by preeclampsia.[514] Maternal serum CRH levels are increased and CRHBP decreased in pregnancies complicated by preeclampsia, and measures of maternal CRH and CRHBP may be predictive of the risk for development of preeclampsia.[499]

CRH at relatively high concentrations can also act on vascular smooth muscle and endothelial cells to increase peripheral vasodilation and thus lower arterial blood pressure.[515] Although a physiologic role for CRH on maternal cardiovascular function remains to be determined, it has been shown that the hormone is a potent dilator of and thus may contribute to the regulation of the uteroplacental circulation and vascular tone.[516]

In addition, CRH and Ucn stimulate processing of placental POMC and thus expression of ACTH, β-endorphin, and α-MSH.[517] Placental ACTH is localized primarily to the cytotrophoblast in the first trimester and the syncytiotrophoblast thereafter, and expression and levels of placental ACTH increase with advancing gestation. However, placental ACTH does not appear to contribute significantly to maternal ACTH levels. Thus, placental ACTH either acts locally and/or is secreted into the fetus where it has been shown to interact with a family of five melanocortin receptors (MC1R–MC5R) expressed by various fetal tissues to potentially modulate fetal development in rodent models.[518] Moreover, the placenta synthesizes the opioid peptides, enkephalin and dynorphins 1–8 and 1–13.[255,519] Maternal serum levels of enkephalin are similar in pregnant and nonpregnant women, whereas levels of dynorphin are elevated primarily late in gestation. Although maternal levels of β-endorphin have been shown to either increase or not change during pregnancy, placental expression is increased by the stress of delivery.[255] While the specific roles of these opioids on placental function remain to be elucidated, the mu (μ), kappa (κ), and delta (δ) receptors that bind the endorphins, enkephalins, and dynorphins are expressed on placental cell membranes.[520] The κ receptors appear to have the highest affinity for the opioid peptides and thus elicit the most potent effects on aspects of placental function, which include release of hCG in culture.

Gonadotrophin-Releasing Hormone

GnRH is a decapeptide synthesized by the hypothalamus, and two isoforms, GnRH1 and GnRH2, are expressed by human placental cytotrophoblasts and the syncytiotrophoblast.[39] The gene for GnRH1 is located on chromosome 8p11.2–p21 and encodes a 92-amino-acid precursor protein that contains the GnRH decapeptide as well as a signal sequence, a GKR-processing sequence, and a GnRH-associated peptide.[488,521] The gene for GnRH2 is located on chromosome 20p13, is 70% homologous to the GnRH1 gene, and encodes a pre-prohormone and comparable signal and GKR sequences and a decapeptide that differs from GnRH1 in three amino acids. Both GnRH isoforms signal via activation of the GnRH receptor 1 (GnRHR1), which has been localized to human placental cytotrophoblasts, the syncytiotrophoblast, and the decidua.[522,523] The gene encoding GnRHR1 is located on chromosome 4q13.2–21.1 and encodes a GPCR that lacks an intracytoplasmic COOH terminal tail important for receptor desensitization as well as internalization and recycling. A second GnRHR isoform (GnRHR2) has been isolated and the gene localized to chromosome 1q12. However, this receptor does not appear to be functionally active

in humans, and thus GnRH1 and -2 signal through GnRHR1.

Although levels of GnRH appear to be unchanged during the course of gestation, placental mRNA and protein expression of GnRHR1 mirrors that of βhCG and thus increases progressively during the first 8–9 weeks and decreases progressively thereafter.[522] Those factors that increase the release of GnRH from the hypothalamus (e.g., depolarizing and cAMP stimulatory agents, activin A, and E_2) also stimulate placental GnRH secretion.[524] A number of factors appear to regulate GnRHR1 expression, notably GnRH1, activin, and inhibin, and response elements for these factors have been identified in the promoter region of the gene.[255,488]

Placental GnRH regulates βhCG expression,[525] and GnRH1 appears to be the more physiologically relevant in this regard as it has a much higher affinity for GnRHR1 and can stimulate hCG secretion for up to 24 h.[526] GnRH may also have a role in decidualization in early pregnancy, since it induced apoptosis in human decidual stromal cells, an effect mediated by growth arrest DNA damage-inducible gene 45α and MAPK signaling.[523] GnRH1 and GnRH2 also may play a role in EVT invasion[527] via modulation of MMPs.[488,528,529]

Kisspeptin

Kisspeptins comprise a family of proteins derived from the Kiss1 gene, which when translated yields a 145-amino-acid peptide known as kisspeptin 145 or Kiss1. Kiss1 can be further transformed to yield Kiss14, Kiss13, and Kiss10 and the 54-amino-acid peptide metastatin (kisspeptin 54) originally shown to inhibit cancer metastasis.[530] Kiss1 mRNA and protein are highly expressed by the placental syncytiotrophoblast, cytotrophoblasts, and decidua.[531] The kisspeptin receptor, formerly known as the orphan G protein-coupled receptor (GPR54), is expressed in the human hypothalamus and the placenta.[531,532] Placental Kiss1 and GPR54 mRNA levels are elevated in the first trimester and markedly increased in both preterm and postterm, as well as preeclampsia pregnancies,[533–535] while plasma kisspeptin levels exhibit a progressive rise during normal pregnancy.[536]

Kisspeptin modulates GnRH neuronal excitability, function, and expression and thereby influences the hypothalamic–pituitary–gonadal axis and the onset of puberty. Thus, subjects with loss-of-function mutation of the GPR54 have idiopathic hypogonadotropic hypogonadism.[530,532,537] Within the placenta, however, Kiss1 has also been shown to inhibit migration of trophoblast cells in culture,[538] and placental trophoblast kisspeptin expression is low in women with a history of recurrent miscarriage.[531] Recent studies have described the signaling events that are activated by Kiss10 interaction with GPR54 and that apparently involve interaction and cross-talk with the EGF receptor.[539]

Neuropeptide Y

NPY is a 36-amino-acid peptide that is abundantly expressed in the decidua, fetal membranes, and placental cytotrophoblast and intermediate trophoblast,[540] as well as the central and peripheral nervous system. Maternal serum levels of NPY are greater in pregnant than nonpregnant women and remain relatively stable or increase slightly during gestation.[487] Serum NPY levels drop rapidly postpartum, and thus the placenta appears to be a major source of NPY in maternal serum. The two NPY receptor subtypes, NPY1R and NPY3R, are localized to placental syncytiotrophoblast microvilli.[541]

NPY appears to have a role in stimulating placental trophoblast CRH synthesis and release[540] by modulating the phospholipase C–inositol triphosphate receptor axis and calcium calmodulin kinase II activity.[542] NPY may also participate in the regulation of several physiologic processes, including stimulation of food intake, inhibition of insulin release, modulation of gastrointestinal motility, basal secretion of pituitary LH,[543] and cascade of events leading to labor and delivery, although its roles in these processes during pregnancy remain to be clearly defined. However, maternal NPY levels are increased in preeclampsia[544] and, like changes in maternal leptin and other adipokines, may represent a compensatory response in the presence of placental dysfunction.

Oxytocin

Oxytocin is a 9-amino-acid peptide that is transcribed from the oxytocin gene located on chromosome 20p13. Oxytocin is synthesized by or released from the supraoptic nucleus and PVN of the hypothalamus, the axons of which terminate in the posterior pituitary gland. Oxytocin is also produced in the placental syncytiotrophoblast, decidua, amnion, and chorion.[545] Oxytocin mRNA levels in intrauterine tissues are increased in association with spontaneous labor.[545] Maternal serum oxytocin concentrations exhibit a circadian rhythm and are highest at night coinciding with nocturnal spontaneous uterine activity. E_2 appears to be a major regulator of oxytocin expression, particularly in the decidua and chorion.[546] Placental oxytocin expression is also increased by placental CRH, as well as activin and prostaglandins,[547] factors that are part of the cascade of events that appear integral to parturition. The oxytocin receptor is abundantly expressed in myometrium and other intrauterine cells, including the decidua and chorion leavae, where levels are increased by E_2 and decreased by P_4.[548] However, the relative contribution of placental oxytocin to the onset of

labor, particularly the relationship to pituitary oxytocin release during the expulsive phase, is unclear.

Chromogranin A

Chromogranin A is a 49-kDa glycoprotein of the neuroendocrine system that is typically increased by stress, including hypoxia.[489,549] Chromogranin A is colocalized within and co-released from storage granules along with NPY and catecholamines.[549] During pregnancy, chromogranin A is also expressed by the trophoblast, decidua, and fetal membranes, and maternal serum levels exhibit a progressive rise with advancing human gestation.[550]

Plasma chromogranin A levels are higher in the fetus than in the mother and are elevated by the process of spontaneous labor.[551] Therefore, it has been postulated that chromogranin A may inhibit catecholamine and NPY release to counteract the vasoconstrictory effects of the latter peptides at the time of delivery.[551]

Neurokinin B

The placenta also expresses NKB, which belongs to the tachykinin family of signaling peptides, such as substance P, that function in the central nervous system as excitatory neurotransmitters for nociception.[552] The 10-amino-acid NKB peptide is abundantly expressed by the syncytiotrophoblast and secreted into the maternal circulation,[553] where levels increase with advancing gestation.[554] The 3G protein-linked NKB receptors (NKBR1–3) are also expressed in human placenta and umbilical vein endothelial cells, as well as hypothalamic neurons.[555]

Mutation of the genes encoding either NKB or NKBR3 causes normosmic isolated hypogonadotropic hypogonadism (niHH), indicating the importance of NKB in human reproductive function and development.[556–558] In contrast to the infertility associated with the null mutation of NKBR3 (Tacr3$^{-/-}$) in humans,[552] Tacr3$^{-/-}$ mice exhibit normal fertility.[559] Consistent with the role of NKB on the hypothalamic–pituitary–gonadal axis in humans, Plant and coworkers[560] showed that administration of NKB to gonadectomized rhesus monkeys elicited robust GnRH-stimulated LH pulses. NKB has also been shown to modulate other physiologic processes, including water homeostasis,[561] pulmonary inflammation,[559] and cognition.[562] NKB may also enhance placental vasodilation via a receptor-mediated mechanism independent of nitric oxide. Finally, maternal levels of NKB are increased in women with preeclampsia, which could reflect a compensatory increase to compromised uteroplacental blood flow.[553]

Monoamines

The placenta also expresses the enzymes that catalyze the synthesis, as well as metabolism, of epinephrine, norepinephrine, dopamine, and serotonin.[563,564] Moreover, receptors and transporters for these factors are localized in the placental trophoblast and altered in pregnancy complications, including preeclampsia.[563]

Endothelin (ET)

Endothelins (ETs) are 21-amino-acid peptides produced by several cell types (e.g., fibroblasts and macrophages, and notably endothelial cells). Three isoforms, ET1–3, have been identified and are encoded by three separate genes. ET1 is synthesized as a 212-amino-acid pre-peptide, which is processed to a 38-amino-acid precursor (Big ET1) that is subsequently cleaved into the mature ET1 by a specific ET-converting enzyme, termed ECE1.[565] PreproET1 mRNA and ET1 protein are synthesized by placental endothelial cells, as well as villous trophoblast and amniotic epithelium.[566] ECE1 is expressed in endothelial cells lining the vessels of the maternal basal plate and fetoplacental vessels.[567,568] Placental ET1 receptor expression appears to be highest in early gestation.

ET1 is a potent vasoconstrictor of the human placental vasculature and thus in conjunction with other factors likely plays a role in control of uteroplacental blood flow.[569] There also is considerable evidence that placental ET has a role in the pathology of preeclampsia.[570] For example, placental ET1 expression[571] and circulating ET1 levels[572,573] are higher in women with preeclampsia. Granger and colleagues[574–577] have used the reduced uterine perfusion pressure (RUPP) rat as a model for preeclampsia. In the RUPP rodent, which exhibits hypertension, renal injury, proteinuria, and endothelial dysfunction, the ischemic placenta produced factors that increased vascular ET1 production, and ET1 mediated the onset of hypertension induced by administration of TNFα or the soluble Flt-1 VEGF receptor.[574]

Adrenomedullin

AM is a 52-amino-acid peptide with homology to CGRP. Human AM is processed from preproAM and proAM via enzymatic amidation. Originally discovered in 1993 in human adrenal pheochromocytoma, AM mRNA and protein are also expressed in the syncytiotrophoblast, villous cytotrophoblast, EVTs, and chorio decidua.[578,579] Maternal levels of AM are threefold higher in pregnant than nonpregnant women and rise during the course of human gestation.[580]

Homozygous AM$^{-/-}$ mice produce embryos with little vascularization of the yolk sac and fetal death

at midgestation caused by cardiovascular defects.[581] Administration of an AM antagonist caused FGR and defective blood vessel development in the placental labyrinth.[582] AM decreases vasomotor tone in isolated perfused placental vessels previously constricted with thromboxane.[583] Thus, AM may participate in the maintenance of low resistance of the placental vascular bed by binding to a GPCR, stimulating the nitric oxide cGMP pathway in endothelial cells, and activating cAMP in smooth muscle cells.[584] Although AM mRNA expression in the trophoblast, amnion, and chorio decidua was increased in patients with preeclampsia,[585] maternal AM levels were unaltered, increased, or decreased in other studies of pregnancies with preeclampsia.[586]

Calcitonin Gene-related Peptide

CGRP, a 37-amino-acid neuropeptide originally shown to influence nociception and modulation of the autonomic system, also exhibits potent vasodilatory effects.[587] CGRP mRNA and protein are expressed in the syncytiotrophoblast, villous vascular endothelial cells, EVTs, and decidua.[588–590] CGRP is secreted into the maternal circulation, where levels progressively increase with advancing gestation.[589] CGRP acts via a seven-transmembrane GPCR, which is modified by receptor activity-modifying proteins (RAMP1–3), and is localized to the placental syncytiotrophoblast microvillous and BMs, as well as the EVTs and the myometrium, where levels are highest prior to labor.[255]

Homozygous CGRP receptor-null mice exhibit cardiovascular defects, hydrops fetalis, and embryonic death at midgestation,[591] indicating the importance of CGRP for embryonic and fetal development. A major function of CGRP in pregnancy appears to be modulation of uteroplacental vasoactive tone.[592] Interestingly, in pregnant rodents, the induction of hypertension by administration of L-NAME, which blocks nitric oxide production, was prevented by concomitant administration of CGRP.[593] Moreover, Yallampalli and colleagues[590] and others[594] have shown that CGRP induced human vascular endothelial cell proliferation and migration and capillary-like tube formation in a dose- and time-dependent manner. The authors[590] suggested, therefore, that CGRP has a role in placental angiogenesis and neovascularization. It has also been suggested that CGRP may play an important role in the etiology and/or progression of preeclampsia, although maternal CGRP levels were either decreased[588,589] or unaltered[595] in preeclampsia.

Parathyroid Hormone-related Peptide

PTHrP is a 141-amino-acid protein that shares substantial sequence homology with parathyroid hormone (PTH). PTHrP was originally purified from tumors associated with hypercalcemia, and thus circulating levels are typically low except in such clinical conditions. Following enzymatic cleavage, several forms of the protein exist, including PTHrP1–34 and 67–86. PTHrP1–34 protein is expressed in the syncytiotrophoblast, whereas PTHrP67–86 is expressed primarily in endothelial cells of placental villous capillaries.[596] Maternal serum levels of PTHrP are elevated in pregnancy and appear to be maximal in late gestation. PTHrP binds to the PTH receptor, which is also expressed in the placenta and myometrium.

PTHrP-deficient mice show a reduced maternal–fetal calcium gradient and skeletal dysplasia, and they die at birth,[597] in contrast to PTH-null mice in which placental calcium transfer was unaltered.[598] In rats in which fetal growth was reduced by uteroplacental restriction, fetal and placental calcium homeostasis was disrupted and placental PTHrP expression increased, presumably to compensate for impaired placental function.[599] Finally, PTHrP also elicits a potent vasodilatory effect on the uteroplacental circulation and inhibition of uterine contraction.[592,600]

ADIPOKINES

Over the past decade, several factors, including leptin, adiponectin, resistin, and ghrelin, have been isolated from adipose and other tissues and shown to be important to metabolic homeostasis and satiety. In addition to PL (discussed in this chapter), these factors, termed adipokines, also play an important role in the metabolic adaptations that characterize human pregnancy, notably the increase in maternal insulin resistance with advancing gestation. A review of the expression, physiology, and interaction of these adipokines in controlling food intake, metabolism, and insulin sensitivity has been published.[601] Therefore, our emphasis will be to summarize the nature, expression, and metabolic role of these factors in pregnancy; their potential impact on placental function; and their secretion in pregnancies complicated by FGR, obesity, and hypertension or preeclampsia.

Leptin

Leptin is a 146-amino-acid, 16 kDa protein hormone, discovered in 1994,[602] that regulates food intake, energy expenditure, and metabolism and thus body weight.[603] Originally thought to be primarily the product of adipose tissue,[602] leptin is also expressed by the placenta as well as numerous other tissues, including muscle, liver, brain, and ovary.[604–606]

The actions of leptin are modulated by the leptin receptor, which is a member of the cytokine receptor superfamily that includes receptors for interleukins,

prolactin, growth hormone, and erythropoietin.[607,608] As a consequence of differential splicing, there are four known isoforms characterized by the length and sequence of the cytoplasmic terminal and classified as long (HLR_L) or short (HLR_S), the latter composed of isoforms with cytoplasmic regions of 5, 15, or 67 amino acids.[607,608] In all receptor isoforms, the extracellular ligand-binding and transmembrane domains are identical, as are the first 29 amino acids of the cytoplasmic region. All isoforms contain a "box 1" region involved in binding JAKs. However, the long form also has a "box 2" site that binds STAT, and thus only HLR_L activates JAK–STAT. HLR_{S15} activates and thus signals via MAPK.[608,609] Finally, soluble receptors (sol-HLRs) generated by proteolytic cleavage of membrane-bound HLRs are released into and thus detected in the peripheral circulation.[610] Because these soluble receptors can sequester and thus reduce the availability of leptin, they are thought to play a functional role in the leptin resistance characteristic of some forms of obesity as well as pregnancy.[605]

Maternal serum concentrations of leptin are elevated very early in gestation and continue to rise throughout the remainder of pregnancy, peaking during the second and third trimesters.[611–613] That the placenta is a major source of maternal circulating leptin is supported by studies showing that leptin mRNA and protein are expressed in placental syncytiotrophoblast throughout gestation, as well as by the cytotrophoblast in early gestation, and that maternal serum levels become elevated before any increase or change in maternal adiposity and return to baseline rapidly after delivery.[605,611,612,614,615] Placental leptin mRNA levels are much higher in early than late gestation and thus, as discussed further here, placental leptin expression and maternal serum levels appear to be regulated by the hormonal milieu of pregnancy and not by trophoblast mass.[605] Maternal adipose, however, remains a source of leptin in pregnancy. Thus, regardless of the stage of gestation, maternal leptin levels are considerably higher in overweight pregnant women (Body Mass Index (BMI) > 26 kg/m^2), whereas placental leptin expression is similar in obese and lean women.[601]

In addition to expression of HLR by maternal tissues (e.g., HLR_L in the hypothalamus and HLR_S in the liver and adipose), the placenta as well as the amnion, chorion, and umbilical vasculature express mRNA and protein for both HLR_L and HLR_S. Receptors are expressed throughout the course of gestation, and proteins are localized to the syncytiotrophoblast, the EVTs, and fetal vascular endothelial cells.[616] Levels of the sol-HLR in maternal serum appear to be elevated in pregnancy. As a consequence, the concentrations of maternal unbound leptin remain elevated but are not further increased during the second and third trimesters.[617] Therefore, it has been suggested that the level of leptin available to bind HLR_L in the maternal hypothalamus is not further increased and/or that this very high level of leptin downregulates hypothalamic HLR_L expression and function.[605]

Leptin is encoded by the *lep* gene located on chromosome 7q31, and transcription in adipose is increased by glucocorticoids, insulin, and E_2 and decreased by β-adrenergic agonists.[618,619] In addition to a TATA box, three motifs including C/EBP sites and an SP1 site, as well as consensus half sites for ER and a hypoxia response element (HRE) in the promoter, contribute to leptin transcription.[619] Although the same promoter is used by adipose and placenta, the placenta utilizes a specific enhancer composed of three protein-binding elements (PLE1–3) 1.9 base pairs upstream and localized within a medium reiteration frequency repeat (MER11) element of the *lep* gene.[618] Interestingly, MER11 elements are often found in the human genome but are not present in the murine genome, which likely explains why the human but not the murine placenta expresses leptin mRNA.[619] It is important to note that the proteins binding the PLE3 motif in the *lep* gene are placenta specific. Moreover, E_2 appears to be the major regulator of placental *lep* gene expression[606,620,621] and likely functions via both nuclear and membrane-associated ERα-induced MAPK and Akt pathways (Figure 40.17).[369,370] In addition, as discussed in the section on hCG, Maymo and colleagues[369,371] and others[622] have shown that hCG stimulates placental leptin expression in a complex manner mediated by cAMP, MAPK,

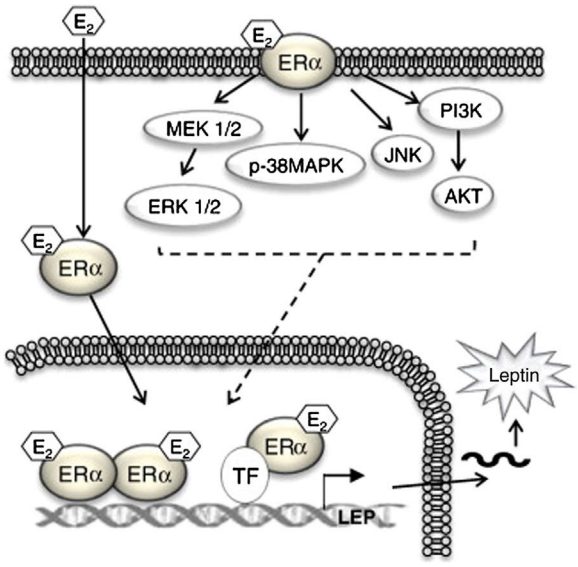

FIGURE 40.17 Proposed model of estradiol (E_2) mechanisms involved in induced leptin expression in trophoblastic cells. E_2 action involves nuclear and membrane-associated estrogen receptor alpha (ERα). The activation of several signaling pathways in response to E_2 regulates leptin expression. Another transcription factor (TF) could also be involved. *Source: Reprinted with permission from Ref. 370.*

and cAMP–Epac signaling pathways. Finally, placental leptin mRNA levels are elevated by glucocorticoid[623] and hypoxia[624,625] and thus *lep* gene expression may also be responsive to HIF-1α.[619,626]

Tissue-specific regulation of leptin expression is also known to be epigenetically mediated through DNA methylation at a tissue-specific region in the *lep* gene promoter.[619,627,628] Moreover, there is an inverse relationship between DNA methylation in this region and gene expression. For example, in adipose, DNA methylation is low and leptin gene expression high, whereas in liver DNA methylation is high and *lep* gene expression low. Thus, the degree of methylation of the *lep* gene in the placenta could also play an important role in leptin expression in normal pregnancy, as well as in pregnancies complicated by FGR, diabetes, and preeclampsia.[619,627]

In addition to regulation of satiety, leptin has other metabolic functions, including the suppression of pancreatic β cell insulin secretion, enhancement of adipocyte glucose utilization and lipolysis, increase in insulin sensitivity, and uptake of sugar by the small intestine. However, leptin appears to participate in other physiologic processes as well, including angiogenesis, hematopoiesis, and osteogenesis, as well as in neuroendocrine, immune,[606,629] and reproductive function (e.g., puberty, oocyte maturation, and implantation).[605]

Importantly, the primary physiologic role of leptin to suppress food intake is reduced during pregnancy, presumably to assure nutrient availability for fetal growth and maternal metabolic homeostasis. The hyperphagia of pregnancy is associated with or caused by leptin resistance and disruption of the leptin receptor signaling pathway in the hypothalamus and/or changes in placental hormone (e.g., PL secretion).[630] In addition to effects on maternal tissues, leptin has been shown to modulate several aspects of placental function, including stimulation of proliferation and suppression of trophoblast apoptosis, upregulation of syncytiotrophoblast system A sodium-dependent neutral amino acid transport activity critical to fetal growth, as well as enhancing placental lipid catabolism, nitrous oxide production, and the secretion of hCG, prostaglandins, and pro-inflammatory cytokines.[606,615,631–634]

Adiponectin

Adiponectin is a 248-amino-acid, 30 kDa protein that enhances insulin sensitivity and reduces hepatic glucose production, and thus hypo-adiponectinemia is typically associated with insulin resistance.[635,636] Although this metabolic hormone is abundantly and almost exclusively produced by adipose, several studies have shown that the placenta also expresses adiponectin mRNA.[601,615,636] The full-length protein (fAD) consists of an N-terminal sequence, a collagen domain, and a C-terminal globular domain and can multimerize to form several stable low-, medium-, and high-molecular-weight complexes. Importantly, fAD is processed to generate a 16.5 kDa truncated globular protein (gAD) that is an active form of adiponectin.[636] Low plasma adiponectin levels that are characteristic of diseases, such as type 2 diabetes and gastric cancer, not only reflect reduced adipose gene expression but also are often associated with single-nucleotide polymorphisms (SNPs) in the adiponectin gene located on chromosome 3q27.[637]

The adiponectin receptors, ADIPOR1 and -R2, are expressed in multiple tissues. ADIPOR1 is ubiquitously expressed but predominates in skeletal muscle and binds gAD with high affinity. In contrast, ADIPOR2 is primarily localized to liver and has higher affinity for fAD than gAD.[638] The human placenta also expresses mRNA for both receptors, although only the ADIPOR2 protein has been detected and localized to the syncytiotrophoblast.[615,639] In humans, maternal serum adiponectin levels are elevated very early in gestation and then decline and remain relatively constant throughout the remainder of pregnancy. Serum levels in maternal circulation during the second half of gestation are comparable to or slightly lower than respective values in nonpregnant women.[640–642] In contrast, adiponectin levels in the fetus are markedly increased and correlated with gestational age.[643] Fetal adipose, as well as cells of skeletal muscle and intestinal wall, express adiponectin, which is thought to facilitate fetal growth via its ability to increase insulin sensitivity.[644]

As recently reviewed,[615,636] the physiologic roles of adiponectin on placental function remain to be definitively established. However, in vitro studies have shown that adiponectin can decrease trophoblast proliferation; enhance hCG secretion; promote trophoblast MMP expression, angiogenesis, and cell migration; modulate inflammation; as well as increase basal system A activity and antagonize insulin-stimulated system A activity and phosphorylation of the insulin-receptor substrate.[636,645–647] Interestingly, lower DNA methylation of the adiponectin ADIPOQ gene promoter on the fetal side of the placenta was correlated with high maternal glucose and adiponectin levels and lower DNA methylation on the maternal side of the placenta associated with a higher insulin resistance index.[648] The authors suggested that these placental epigenetic adaptations have the potential to induce sustained glucose metabolism changes in the mother and offspring later in life. Finally, recent studies have also shown that insulin signaling and insulin-regulated amino acid transporter expression and activity were attenuated in placentas of pregnant mice chronically infused with fAD.[649]

Resistin

Resistin, a protein hormone composed of two 92-amino-acid disulfide-linked polypeptides, impairs adipose cell glucose uptake, increases blood glucose levels, and thus decreases insulin sensitivity.[650,651] Originally isolated from adipocytes in animal models and in nonfat cells of adipose depots in humans, resistin mRNA and protein are also expressed by the human placenta and localized to the syncytiotrophoblast and EVTs in early gestation and the syncytiotrophoblast in late gestation.[652] Maternal serum levels in the first and second trimesters are relatively constant and comparable to values in nonpregnant women. However, resistin levels and placental mRNA expression are increased by the third trimester.[653] Because adipose resistin expression remains unchanged during pregnancy,[629,653] placental production is likely a major source of resistin in the maternal circulation where in conjunction with PL this factor is thought to play an important role in the marked increase in insulin resistance with advancing gestation.[601] The factors controlling expression of the gene encoding resistin, which is localized to chromosome 19p13.2, remain to be elucidated.

Studies have shown that resistin increases migration and invasiveness of BeWo cells via upregulation of MMP2 activity and expression, as well as suppression of tissue inhibitors of metalloproteinases 1 and 2 (TIMP1/2).[654] Resistin enhances endothelial cell tube formation as well as expression of VEGF, the VEGF receptors, and MMP1 and -2, and thus may also play a role in villous angiogenesis.[615,654]

Ghrelin

Ghrelin is a 28-amino-acid peptide hormone produced by mucosa cells of the stomach as well as other tissues, including the intestine, pancreas, kidneys, and placenta.[655] Ghrelin circulates bound to lipoproteins, and about 5–10% of the hormone has a fatty acid modification (octanoate residue) at serine 3 catalyzed by the enzyme ghrelin-*O*-acyl-transferase. Acyl ghrelin (AG) binds to the growth hormone secretagogue receptor (GHS-R1a) to elicit stimulation of pituitary GH secretion as well as appetite. Once in blood, active ghrelin is rapidly deacylated by enzymes that cleave the octanoate residue to form des-acyl-ghrelin (DAG), which accounts for up to 90% of the hormone circulating in plasma.[656] Recent studies suggest that DAG acting via pathways yet to be identified alone and/or in conjunction with AG stimulates adipogenesis. Importantly, hyperglycemia and insulin resistance are independently associated with a decrease in ghrelin expression.

In pregnancy, ghrelin (AG plus DAG) levels in maternal serum and mRNA expression in placenta are highest in early to midgestation, decline after the second trimester, and remain relatively low during the third trimester.[657,658] It also appears that maternal levels of AG decline in late gestation.[659,660] Although maternal levels of ghrelin are not significantly correlated with BMI or insulin levels, the decrease in serum ghrelin after midgestation occurs when maternal weight and fasting insulin levels (i.e., insulin resistance) are increasing.[658]

The placenta expresses the functional GHSR1a ghrelin receptor, and in placental JEG3 cells ghrelin stimulated proliferation and decreased caspase 3 activity as well as the production of progesterone, but had no effect on hCG expression.[661] The factors regulating placental ghrelin expression remain to be elucidated. However, it has been shown that women carrying the 51Q allele of the ghrelin gene have a fivefold lower risk of developing gestational diabetes.[662] 51Q is also protective against metabolic syndrome where glucose intolerance is a factor.[663] Presumably, the R51 residue is a site for proteolytic cleavage to produce mature ghrelin, and the 51Q allele apparently leads to lower blood levels of ghrelin. Thus, the protective effect of 51Q may reflect decreased levels of ghrelin, which would facilitate and/or permit increased pancreatic release of insulin.

Adipokine Expression in Pregnancy Complications

Preeclampsia

Maternal serum leptin levels are increased in pregnancies complicated by preeclampsia.[601,641,664,665] This increase was similar in obese and nonobese mothers,[665] and presumably reflects increased production of leptin by the placenta.[641,666,667] Maternal leptin levels and placental mRNA are further increased in preeclamptic pregnancies complicated by FGR[665,668] and small for gestational age neonates.[669] Upregulation of placental leptin expression in preeclampsia may reflect a compensatory response of and need for increased nutrient delivery to an underperfused placenta[641] and/or increased *lep* gene transcription in response to hypoxia and HIF-1α.[619] Moreover, recent studies have shown that CpG sites downstream of the transcription start site of the placental *lep* gene are significantly hypomethylated in pregnancies complicated by early-onset preeclampsia.[619] In addition, because *lep* gene expression was less skewed and thus more biallelic, it appears that the loss of normal monoallelic expression associated with hypomethylation in early-onset preeclampsia may lead to overall increased *lep* gene expression. Interestingly, however, hypomethylation was not detected in placentas of patients with late-onset preeclampsia or in normotensive women with FGR.[619]

Maternal levels of adiponectin and resistin are either increased[641,665,670] or decreased[642,653] in preeclamptic pregnancies. The differences in adiponectin may reflect

maternal body weight and BMI, since preeclamptic women with normal BMI have elevated adiponectin, whereas obese women presumably with exaggerated insulin resistance have decreased adiponectin.[642,671] Although resistin levels appear to be independent of BMI, differences in preeclamptic women may reflect dysfunction in other maternal organs (e.g., kidney).[672] Finally, the role and regulation of placental and adipose expression of these factors in preeclampsia remain to be determined.

Gestational Diabetes Mellitus (GDM)

It is generally accepted that the expression and maternal serum levels of leptin, adiponectin, resistin, and ghrelin are dysregulated in pregnancies complicated by gestational diabetes mellitus (GDM).[673–675] However, results are inconsistent and presumably reflect differences in maternal BMI and the extent and/or time (during or before pregnancy) of onset of metabolic dysfunction.[601] Thus, a number of studies clearly indicate that maternal leptin levels as well as placental leptin mRNA expression are increased in women with GDM, while others have shown that leptin was either reduced or unchanged.[676,677] Similarly, while risk of development of diabetes in pregnancy is increased in patients in which adiponectin levels are reduced in early gestation, maternal adiponectin levels in the second half of gestation were unaltered or increased in GDM.[677–679] Interestingly, using glycemic clamp procedures, Gibson et al.[680] showed that maternal leptin and adiponectin levels were not altered by insulin or glucose in GDM pregnant women studied in the second and third trimesters. In these same patients, however, levels of DAG but not AG were decreased by glucose and insulin. These findings contrast with studies showing that maternal AG levels are unaltered, whereas those of DAG are elevated, in patients with GDM. Finally, maternal levels of resistin, which increase normally in late gestation, have been shown to be similar, further elevated, or decreased in GDM.[681–684]

In summary, with advancing gestation placental expression and maternal serum levels of leptin, resistin, hPL, and placental GH increase, whereas those of adiponectin and ghrelin decrease, an overall pattern of hormone expression that would facilitate maternal satiety and decrease insulin sensitivity to ensure adequate maternal nutrient (e.g., glucose) supply to the fetus for growth and maternal adipose deposition for maternal metabolic homeostasis. Leptin and adiponectin may also act via their respective receptors expressed in placental cells to regulate aspects of trophoblast function, including expression and/or activity of nutrient transporters important to fetal growth.

Furthermore, it appears that placental *lep* gene transcription, mRNA levels, and maternal serum concentrations are elevated in pregnancies complicated by preeclampsia and further increased in preeclamptic patients with FGR independent of maternal BMI, presumably as a compensatory response to and increased nutrient need of an underperfused placenta and/or hypoxia. Although changes in adiponectin and resistin may also be associated with preeclampsia, results are equivocal and confounded by the apparent impact of BMI as well as renal function on expression of these adipokines. Finally, while evidence clearly shows dysregulation of leptin and adiponectin as well as resistin and ghrelin in GDM, results are highly variable and attributable to the impact of confounding variables, including BMI, the extent of diabetes, the timing of diabetes onset before and/or during pregnancy, as well as potential pregestational treatment modalities. Clearly there is need for additional studies to establish the impact of these multiple adipokines during normal pregnancy and in GDM.

CONCLUSION

Research in placental biology during the past few decades has substantially advanced our understanding of the structure, temporal, and cell-specific expression, actions, and regulation of well-established (e.g., hCG) as well as more recently identified (e.g., adipokines) hormones during pregnancy. The latter investigation, coupled with highly novel technologies to examine cell signaling and gene structure, modification, and function, have further increased knowledge of the mechanisms underpinning the expression, action, and potential involvement of placental hormones in normal as well as abnormal pregnancies. However, it is apparent from examination of these studies that, in many instances, a clear picture does not exist of how the multiple hormones expressed by the mother, placenta, and fetus interact to control pregnancy maintenance, fetal development, and maternal–fetal homeostasis.

An example of this gap in knowledge in placental endocrine biology is how well-established hormones such as PL and IGF interact with more recently identified peptides (e.g., leptin) to control maternal and fetal intermediary metabolism, homeostasis, and growth. Moreover, there is lack of clarity about whether and how the numerous factors recently implicated by in vitro approaches regulate in vivo placental EVT migration, invasion, and remodeling of the uterine arteries. Another challenge in placental endocrine research is to clearly establish how placental E_2, P_4, and the placental hypothalamic-like neural peptides (e.g., CRH and oxytocin) interact in a gestational stage-specific manner to control uterine function critical to the initiation of labor. Follow-up studies of the consequences of alterations in placental and fetal function by experimental paradigms,

or as associated with problem pregnancies, on development of the offspring are also needed.

Several experimental models have been employed to study placental endocrine function and hormone action. Transgenic mouse studies have enabled discovery of the impact of loss of gene function on fetal and organ system development. However, there are significant differences between rodents and humans in placentation, hormone biosynthesis, and endocrine interaction between the mother, placenta, and fetus. Moreover, there appear to be major differences during rodent and human pregnancy in the effect of placental hormones on organ maturation (e.g., the role of placental NKB on gonadal development and function and the ontogeny of and import of placental E_2 on ovarian follicular development).

Epidemiologic and genetic studies of abnormal human pregnancy have yielded important advances on the association and potential impact of changes in placental hormone production on fetal development. However, the very low incidence of genomic defects (e.g., P450arom deficiency) in many cases in human pregnancy and the difficulty for ethical reasons in performing human research limit ability to comprehensively assess physiological and developmental impact. Moreover, although changes in placental function have been shown to be associated with disease states (e.g., preeclampsia), it is difficult to discern whether these alterations precede and thus are involved in the etiology or are the result of the pathological condition, and thus conclusions on cause and effect cannot be made. Emphasis also needs to be made on quantifying protein levels on specific cell populations within the highly heterologous placenta. In vitro approaches with primary and transformed human placental cells are extremely valuable in dissecting out molecular mechanisms and signaling pathways that underlie hormone action and receptor biology. However, with cell culture there is always the potential problem of cell differentiation and applicability in vivo to the human situation.

In vivo studies with nonhuman primates that exhibit patterns of maternal–placental–fetal hormone production, interaction, and development qualitatively similar to those in the human offer valuable and significant opportunities to investigate endocrine interactions and their impact on development of the fetus in utero and of offspring after birth, and most importantly to translate findings to the human. However, the relatively high cost of conducting nonhuman primate research limits its widespread use.

While there is no simple solution to address the limitations inherent to any experimental model in vitro, rodent, nonhuman primate, and human approaches provide individual advantages and, when combined with ever emerging technological advances, provide the best opportunity to advance the concept put forth by Egon Dicszfalusy some 50 years ago, namely, that the maternal–fetal–placental systems function as a unit in which there is interdependence and interaction between the endocrine functions of maternal and fetal EGs and the placenta.

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CHAPTER

41

Immunology of Pregnancy

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INTRODUCTION

The principal role of the immune system is to monitor and defend the integrity of the organism. Immune cells act to recognize and usually repel invaders of all types, from microorganisms to tumors and artificial tissue transplants, so as to prevent disease and contamination of host genetic material. Yet this powerful defense system is overcome during pregnancy in mammals where the fetus is gestated internally. This arrangement, known as viviparity, provides maximum protection from environmental hazards but introduces a critical requirement for circumvention of immune rejection. The embryo and the gestational tissues formed after implantation express "foreign" antigens, including some encoded by major histocompatibility (MHC) genes derived from the disparate maternal and paternal chromosomes. By virtue of deriving 50% of its MHC genes from the genetically nonidentical father, the conceptus is thus termed "semiallogeneic" in relation to the mother. Spermatozoa and oocytes also express specific antigens that can provoke autoimmune responses under some conditions. With these hallmark indicators of "nonself" origin, the male and female gametes and all of the conceptus-derived tissues therefore present a serious challenge to the female immune response.

The surprising ability of mammalian females to permit introduction and survival of genetically different cells during implantation, and to nurture the developing fetus throughout the course of pregnancy, has intrigued immunologists for half a century or more. The mechanisms in place for preventing maternal immune attack on the fetus are generally successful since species continue to propagate. Our knowledge of this special immunological relationship is now substantially advanced, but there are pieces of the puzzle still missing. The imperative for research in this field remains strong, considering the major health burden attributable to reproductive conditions including infertility, miscarriage, preeclampsia, and preterm birth, all of which are thought to be at least partly attributable to origins in the maternal immune response. An emerging area is the immune contribution to early life origins of offspring health—that is, how inflammatory disturbances in the maternal–fetal immune relationship can program development of diseases that become evident after birth, ranging from asthma and allergy to metabolic and cardiovascular disorders, and even mental and cognitive dysfunction.[1]

A Brief History

Various theories have been advanced to explain why maternal immune rejection does not occur, and our understanding of the immunology of pregnancy has grown and shifted substantially over the last 70 years. Three interrelated hypotheses concerning physical separation of the fetus and immune system, attenuated antigenicity of the fetus, and systemic maternal immune suppression formulated by the renowned transplantation immunologist Peter Medawar[2] were the first attempt to formally address the issue. Although these ideas informed and guided research in reproductive immunology through the latter half of the twentieth century,[3] ultimately none have withstood the test of time as a sufficient explanation.

In broad terms, discussions concerning the nature of the immune response to pregnancy have centered around

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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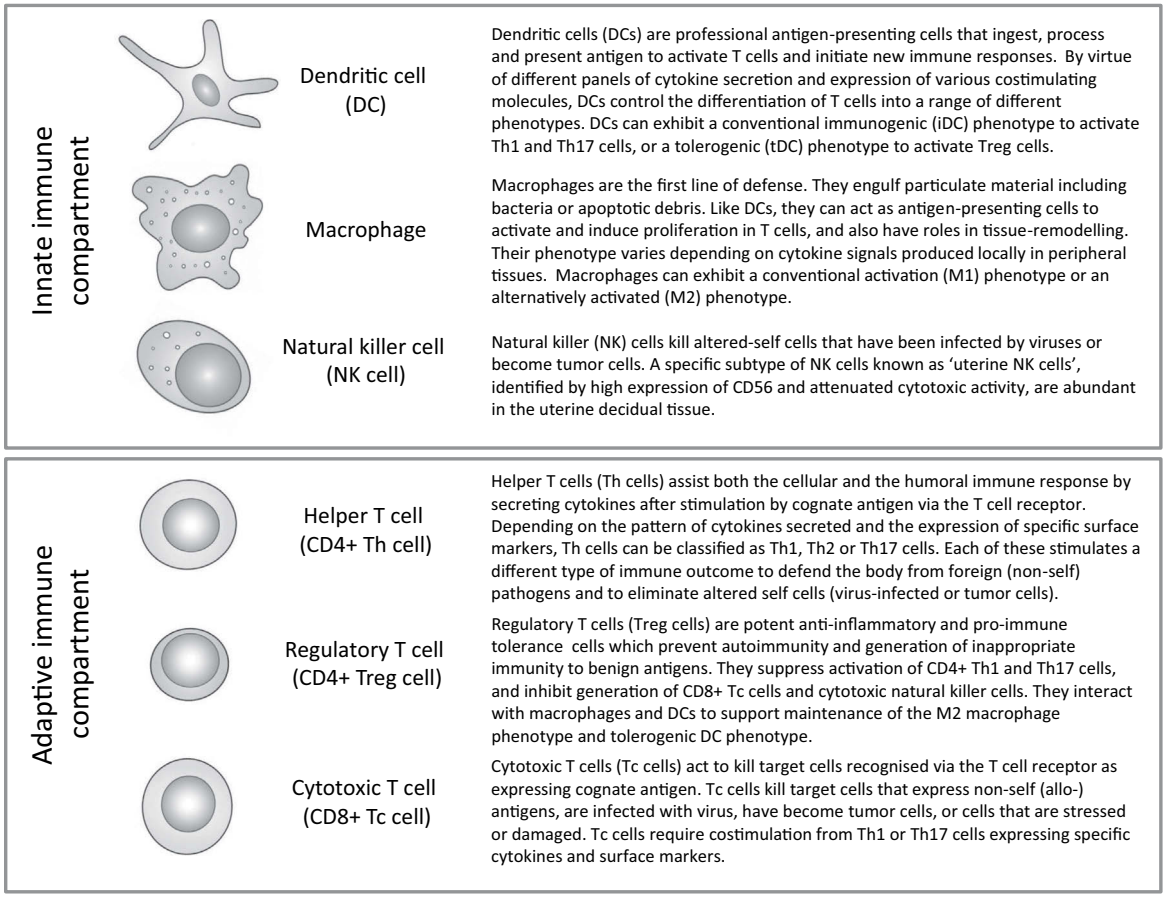
the extent to which the "innate" and "adaptive" immune compartments and their component elements of the immune response are central players. Innate and adaptive immunity are distinguished by their involvement of different leukocyte lineages and separate evolutionary origins. The *innate immune system* is an evolutionarily ancient system consisting of cells that are always present and ready to mobilize and engage with foreign material immediately upon first encounter. The main cellular components of the innate immune system relevant to pregnancy are macrophages, dendritic cells, and natural killer (NK) cells (Box 41.1). The *adaptive immune system*, on the other hand, is a more recently evolved and more sophisticated system of defense involving a repertoire of lymphocytes, which by a mechanism of somatic gene recombination, can express a diverse array of receptor systems with great specificity against individual foreign entities. Components of the adaptive immune system are normally silent; however, when activated, these components "adapt" to the presence of foreign agents by activating, proliferating, and creating potent mechanisms for neutralizing or eliminating the initiating agent. There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B lymphocytes; and cell-mediated immunity, mediated by T lymphocytes. Cells of the adaptive immune response most relevant to pregnancy are the cytotoxic T lymphocytes (Tc cells) marked by CD8 expression, and helper T

BOX 41.1

LEUKOCYTE CELL LINEAGES WITH KEY ROLES IN THE MATERNAL IMMUNE RESPONSE TO PREGNANCY

Leukocyte cell lineages with key roles in the maternal immune response to pregnancy can be assigned to the innate immune compartment (DCs, dendritic cells; macrophages; NK cells, natural killer cells) or the adaptive immune compartment (helper T cells, Th cells; cytotoxic T cells, Tc cells; regulatory T cells, Treg cells). A brief description of the function of each leukocyte lineage is given.



lymphocytes (Th cells) and regulatory T cells (Treg cells), both marked by CD4 expression (Box 41.1).

In the late 1980s Tom Wegmann put forward the formative "Th1/Th2" hypothesis of pregnancy success,[4,5] which influenced reproductive immunology research substantially through the 1990s. This hypothesis was founded in an understanding emerging in the early 1980s, that Th cells can be categorized as Th1 cells, which produce inflammatory cytokines, or Th2 cells, which produce anti-inflammatory cytokines (details are given later in the chapter). Reflecting on observations that T-cell activation can facilitate pregnancy success, together with observations of placental cytokine synthesis and responsiveness, he hypothesized that fetal–maternal immune compatibility might arise from a systemic deviation in the maternal immune response away from potentially destructive Th1 immunity and towards benign Th2 immunity. Because of its manifestation as antibody-mediated rather than cell-mediated immunity, the Th2 response is compatible with fetal survival while Th1 responses appear detrimental. Ultimately it has emerged that the Th1/Th2 hypothesis is an oversimplification of the true state of affairs. Although the principle of immune deviation away from Th1 holds value, Th2 immunity is clearly not a required aspect of maternal acceptance of the fetal allograft.

In contrast to Wegmann's emphasis on T cells and cytokines, other investigators have focused on the extent to which the innate and adaptive compartments of the immune response participate as contributing forces in determining pregnancy success. Drawing on the special anatomical relationship between the placental and maternal tissues in women, the peculiar characteristics of human placental trophoblasts, most notably their altered expression of human leukocyte antigen (HLA) proteins and the vital contribution of an unusual population of NK cells to placentation, Ashley Moffett-King, Joan Hunt, and others have encouraged the view that the immune adaptation to pregnancy is a unique response centered in the innate immune compartment, involving NK cells as opposed to an adaptive response involving T lymphocytes.[6,7]

Current Understanding

In the most recent decade, an integrated model has emerged, acknowledging that both the innate and adaptive immune compartments are involved in the maternal immune adaptation required to avert rejection of the conceptus.[8,9] Importantly, current theories accommodate long-standing evidence that maternal lymphocytes are not ignorant of the embryo, but instead are actively primed to recognize conceptus antigens, as indicated by the presence of activated T cells and antibodies in the blood of pregnant women.[10–12] The most powerful experimental evidence for this comes from T-cell transgenic mice where it is possible to track T cells that specifically react with fetal alloantigens.[13] Recent studies clearly demonstrate that a state of active immune tolerance in the adaptive immune compartment must exist at implantation to prevent antigen-specific immunity towards the invading conceptus.[14] Regulatory T (Treg) cells, a special subset of T cells, have emerged as central players in mediating this tolerance.[15,16] Treg cells are powerful inhibitors of inflammatory type 1 (cell-mediated) immunity that also interact with macrophages and dendritic cells, NK cells, and other cells of the innate and adaptive immune response to suppress inflammatory activation and maintain immune quiescence.[17]

Thus current understanding holds that preventing fetal immune rejection involves a combination of strategies, with multiple overlapping and complementary mechanisms operating for the protection of the fetus. These strategies relate to attenuation, suppression, or evasion of different compartments and different phases of the immune response. Some key aspects of the immune response are circumvented, while others are engaged and persuaded towards immune acceptance, or "tolerance," in order to actively permit the intimate associations between maternal cells and gametes or embryos that characterize oocyte maturation and embryogenesis, implantation, and placental development. Figure 41.1 illustrates the critical point that both mother and fetus cooperate to create the immunological environment that allows fetal growth and development.

The maternal aspect of the fetal–maternal interface features pathways that: (1) diminish the likelihood of activativing adaptive immunity; (2) ensure any immune activation is skewed towards immune tolerance, as opposed to immune rejection; and/or (3) facilitate the tissue remodeling required to support placental development and function. A specific chemokine gene expression profile in decidual cells affords a unique "gatekeeper" role of this tissue in governing leukocyte recruitment. Those leukocytes permitted access to the maternal decidua are associated not only with immune regulation but also feature populations such as uterine NK (uNK) cells that promote structural changes in the decidual vasculature to support placental invasion and development. The macrophages, dendritic cells, and Treg cells that mediate adaptive immune tolerance and inhibit immunity to conceptus antigens in pregnancy also influence vascular dynamics through their potent capacity to suppress inflammation. Soluble molecules that establish and maintain the maternal–fetal interface as a site of immune competence (cytokines and chemokines) and others that have immunosuppressive and immune-deviating properties (hormones and prostaglandins) are also important for sustaining the unique

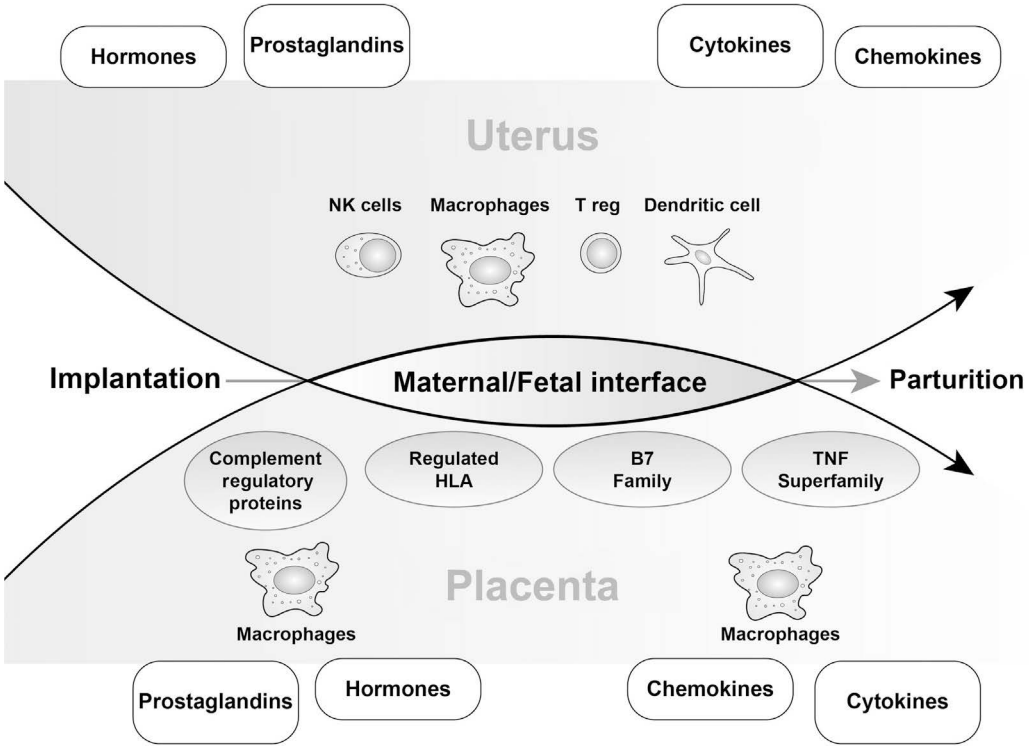


FIGURE 41.1 Multiple mechanisms underlie maternal tolerance of the fetus. Both maternal and fetal mechanisms contribute to successful gestation of the genetically different fetal semi-allograft. Mothers revise the roster of the different leukocyte lineages (macrophages, dendritic cells, NK cells, lymphocytes; see Box 41.1) in the decidua to disallow residence of most antigen-specific immune cells. Production of soluble immune-regulatory molecules including hormones, prostaglandins, cytokines, and chemokines from both uterine and placental cells occurs; the placenta and extraplacental membranes produce many of the same immune-suppressive molecules but also display specially selected members of multi-gene families that bypass or inactivate immune cells. NK cells, natural killer cells; Treg, CD4+ regulatory T cells; HLA, human leukocyte antigen family; TNF superfamily, tumor necrosis factor superfamily; B7, B7 peripheral membrane protein co-stimulator family.

features of the maternal tissues that interact directly with the fetal-placental unit.

Fetal and particularly placental tissues contribute novel mechanisms that include but are not limited to the unique physical structure of the placenta; its restricted expression of MHC transplantation antigens that normally mediate graft rejection; secretion of cytokines, hormones, and soluble MHC antigens that deviate immune responses away from inflammation and towards immune tolerance; high expression of proteins that suppress lymphocyte activation and kill activated lymphocytes; and display of other proteins that protect against cytotoxic antibodies.

In broad terms there are common mechanisms operating in all mammals, but considerable variation exists among species, which relates to different reproductive strategies as well as the structure of the gestational tissues and the extent of their integration and invasion into the mother's uterus and blood supply. The most diverse and sophisticated mechanisms are evident in humans, where maternal and fetal cells intermingle in a type of placentation called hemochorial, and where single pregnancies are the rule. Many of the same evasive strategies prevail in nonhuman primates such as the monkey and the baboon, where placentation is also hemochorial and single pregnancies are usual. In other mammalian species, these two conditions are rarely met; maternal and fetal cells are less intimately associated or multiple fetuses are the rule rather than the exception. Yet many of the mechanisms that ensure successful human pregnancy are present to a greater or lesser degree in these other species, thus broadening the range of informative experimental models and providing insights on the molecular basis of the strategies allowing successful reproduction. In particular, mice are an important experimental model with the benefit of a powerful toolbox of immunological reagents and resources, including many transgenic and knockout genetic models and intervention strategies that allow investigators to define critical molecular and cellular pathways. The value of mouse models is evidenced by the many pathways identified in mice that in turn have proven to be operative in primate and human systems.

In this chapter, features of the female reproductive organs and specific strategies that protect against immune-mediated embryonic loss are described. While the focus is the human, mice and other animals are discussed in the context of comparative biology and/or

their provision of important insights. Key concepts are that:

- Both the mother and the fetus contribute to successful pregnancy.
- Multiple immune evasion and tolerogenic mechanisms are deployed.
- Immune adaptations to permit internal habitation of the fetus depend on ovarian steroid hormones and are initiated prior to and concurrently with implantation.

Major features include specific structural elements of the ovary and uterus that support immune accommodation of the gametes and conceptus; characteristic features of the leukocyte subpopulations that access the uterus and decidua; production of immune suppressive and pro-tolerogenic molecules by both maternal cells and fetal trophoblast cells; restrictions on fetal trophoblast expression of the MHC molecules that provoke graft rejection; and a critical role for T-regulatory cells, which suppress inflammation and inhibit generation of effector T-cell populations capable of harming the fetus. Collectively, these strategies comprise a complex series of mechanisms that permit implantation and facilitate survival and progression of semi-allogeneic pregnancy, while contributing to the quality control processes that maximize reproductive investment and promote a healthy outbred population.

IMMUNOLOGICAL FEATURES OF THE NONPREGNANT UTERUS

In terms of immune competence, the cycling uterus closely resembles other mucosal tissues whose surfaces are exposed to environmental agents and pathogens. But unlike other tissues, the uterus of a reproductive-aged woman undergoes extensive and predictable remodeling due to responsiveness to ovarian steroid hormones, which coordinate preparation each cycle for potential embryo implantation and pregnancy. This remodeling includes substantial changes in the immune cell component, such that the maternal immune adaptation for pregnancy can be viewed as commencing even before conception and embryo implantation.

Hormones and Leukocytes in the Cycling Uterus

Uterine cells expressing specific hormone receptors respond dramatically to cyclic changes in the levels of steroid sex hormones circulating in the blood. As a consequence, major tissue alterations occur in an ordered and chronologically predictable manner. These events are reviewed in detail elsewhere in this volume (see Chapters 24, 25 and 38). In brief, in the early proliferative phase that follows menstruation, estradiols predominate and stimulate proliferation and partial differentiation of the endometrial epithelial cells. At approximately the time of ovulation, estrogens decline and progesterone from the corpus luteum targets the mesenchymal cells of the endometrium to initiate the changes required for receptivity for embryo implantation.

An important aspect of this cycle is that leukocytes respond to the same hormones. There are cycle-related fluctuations in circulating leukocytes in the peripheral blood, and these reflect more overt changes in the reproductive tissues, largely by virtue of regulation of expression of the cytokines and growth factors produced by hormone-stimulated endometrial cells,[18] and also by direct effects.[19] Thus, the hormone-driven cyclic changes include alterations in the immune cell populations in the stroma of the endometrium and changes in their functions that in turn faciliate endometrial receptivity and the possibility of embryo implantation and pregnancy. These changes impact susceptibility to infection with less protection in the progesterone-dominated phase. Dependency of these leukocyte changes on specific sex hormones has been extensively tested and verified. For example, it has long been known that estrogens stimulate an influx of macrophages and other leukocytes into the mouse uterus[20] and that human NK cell populations depend on progesterone for maintenance and proliferation.[6,21]

In the human uterus, both T and B lymphocytes are established in lymphoid aggregates in the uterus just as they are at other mucosal surfaces.[22] Endometrial T cells outnumber B cells, and exhibit a range of different phenotypes. In women, most endometrial T cells express the conventional α/β form of the T-cell receptor (TCR), while fewer cells express the γ/δ form typical of mucosal tissues.[23] The majority express the Tc marker CD8 as opposed to the Th marker CD4, and in addition to their location within aggregates adjacent to glands, they can be found in intraepithelial locations, or scattered singly in the endometrial stromal tissue.[23]

Most importantly for reproductive competence, the uterus contains a large proportion of Treg cells, which have important anti-inflammatory and tolerance-inducing properties. Animal and human studies both reveal variation over the course of the nonpregnant cycle, suggesting ovarian hormone regulation of uterine Treg-cell populations, with a notable estrogen-induced increase at the time of ovulation.[24–26] Rising progesterone levels in the luteal phase are involved in sustaining and expanding the Treg-cell populations, potentially acting via nuclear progesterone receptors[27] to elicit memory T-cell markers, which indicate a committed antigen specificity, and to stabilize their suppressive phenotype.[28]

Macrophages and dendritic cells, which both have key functions in antigen presentation and in governing

the nature of the adaptive immune response, are distributed throughout the endometrial stroma and are numerous in the connective tissue in the myometrium.[20,29] The endometrial stroma also contains large numbers of NK cells, which express a unique phenotype specific to the uterus,[30] including markers that vary with the cycle.[31] The leukocytes in the cycling uterus, including the NK cells,[32] have key functions in host defense, but they are also involved in the extensive tissue remodeling, breakdown, and repair associated with structural changes in the endometrium over the cycle. The decidual response, which commences in the implantation-receptive secretory phase, appears to be facilitated by leukocytes, particularly uNK cells[33] and dendritic cells.[34] Near the end of the cycle, mast cells are particularly prominent[35]; these cells appear to have a specific function in driving menstruation.[36]

Cytokine Regulation of Leukocytes in the Cycling Uterus

The behavior of leukocytes in the endometrium is controlled in large part by the cytokines and chemokines present in the local microenvironment. Different cytokine genes show different spatial and temporal expression patterns, suggesting their independent regulation or sequential activation, by mechanisms that are not fully defined but are likely to involve cross-talk between cytokine transcription factors and steroid hormone receptors. In situ hybridization and immunohistochemical studies demonstrate that uterine epithelial cells lining the luminal cavity and comprising the endometrial glands secrete an extensive repertoire of cytokines. Although leukocytes are often assumed to be the principal source of tissue cytokines, in the endometrium, the range and output of cytokines synthesized by uterine epithelial cells generally meets or exceeds that of the leukocyte compartment.[37]

Control of cytokine production by uterine epithelial cells is principally regulated by the ovarian steroids estrogen and progesterone. These hormones interact with agents introduced into the uterus, including seminal fluid and microbial agents, thereby provoking cytokine production by epithelial cells through Toll-like receptors and other signaling pathways. Estrogens drive synthesis of several important pro-inflammatory cytokines in uterine epithelial cells, giving rise to similar patterns of cytokine production in the peri-ovulatory period in the rodent and human uterine epithelium. Several estrogen-regulated cytokines, including colony stimulating factor-1 (CSF1), granulocyte-macrophage CSF (GM-CSF, CSF2), and tumor necrosis factor (TNF), all increase in abundance over the course of the proliferative phase,[37] together with estrogen-induced interferon-γ (IFNγ).[38] Another key factor involved in mediating immune defense in the uterus is interferon-ε (IFNε), which is induced by estrogen in uterine epithelial cells each menstrual cycle. In mice, IFNε has a major role in protection from *Chlamydia* and herpes simplex virus-2 infection.[39]

By contrast, progesterone has an anti-inflammatory effect on epithelial cell cytokines, suppressing production of GM-CSF, interleukin (IL)-1, and several chemokines. It also acts directly on immune cells with effects similar to those of corticosteroids; at high levels progesterone may act through the glucocorticoid receptor (GR) to downregulate production of inflammatory cytokines.[19,40] In women, a secretory phase decline in GM-CSF, and further increase in CSF1, TNF, and leukemia inhibitory factor (LIF), match patterns occurring early in murine pregnancy.[41–44] This suppression of pro-inflammatory cytokines by progesterone after ovulation is one of the first steps each cycle towards preparing endometrial receptivity and initiating immune adaptation for possible embryo implantation and pregnancy.

Cytokines, in particular the chemokine subfamily,[45] play an integral role in regulating leukocyte recruitment and persistence, by orchestrating the molecular events that promote extravasation of specific populations of leukocytes from the peripheral blood and positioning within the tissue. Attachment and transmigration of leukocytes across the endothelial cell barrier, followed by their movement through the tissue and accumulation in various sites, occurs in response to chemokine gradients. Both steroid hormones and products of the embryo regulate chemokine expression in endometrial epithelial cells and stromal cells.[46,47]

Tightly regulated synthesis of a group of at least nine prominent chemokines is implicated in precisely regulating the spatial and temporal fluctuations in each endometrial leukocyte lineage, with combinations of chemokines at different cycle stages targeting different leukocyte lineages. In this manner, distinct chemokine expression profiles during the mid-luteal phase compared with the premenstrual period can account for the uNK cell recruitment required for pregnancy as opposed to the macrophage, eosinophil, and neutrophil recruitment implicated in tissue breakdown and menstrual shedding.[48] For example, progesterone enhances expression of the chemoattractants IL-8 and CCL2 (monocyte chemotactic protein-1, MCP1), as well as the uNK cell chemokines CXCL10 (IP10) and CXCL11 (ITAC), during the window of implantation.[47,49] A further change in chemokine synthesis occurs in the mid-secretory phase as the endometrium attains receptivity for embryo implantation.[50] Chemokines required for recruitment of Treg cells, including CCL19 (macrophage inflammatory protein-3β, MIP3β) and CCL5 (RANTES), are also expressed in the mid-secretory phase.[51,52] In mice, a similar elevation is induced by seminal fluid exposure at coitus.[53]

Infection can dramatically impact the populations of leukocytes found in the uterus. The absolute numbers and relative proportions of lymphocytes, particularly CD4+ T cells, are increased in the reproductive tracts of pathogen-infected mice and women suffering from sexually transmitted disease,[54,55] with the precise numbers and phenotypes depending on the nature of the pathogen and the stage of progression of the disease. The altered immunological environment and elevated inflammation linked with bacterial and/or viral infection of reproductive tissues is crucial to the pathophysiology of infertility in many women.

Immunology of Oocyte Development

As the ovum develops within the follicle, it is physically sequestered away from immune cells circulating in the blood and residing in the ovarian stroma. The ovary is relatively deficient in T and B lymphocytes but contains an abundant population of macrophages, cells that play critical roles in folliculogenesis, tissue restructuring during ovulation, and both formation and regression of the corpus luteum.[56] Ovarian macrophages generally exhibit features of an alternatively activated mode, presumably as a consequence of exposure to progesterone and progesterone-induced cytokines, which together inhibit classical macrophage activation.[57] Within the developing corpus luteum after ovulation, macrophages have a key role in secreting pro-angiogenic factors that are essential for the rapid angiogenesis that underpins progesterone synthesis and support of pregnancy.[58] In addition, ovarian macrophages phagocytose debris to facilitate removal of atretic follicles and exhausted corpora lutea.

The physical barrier provided by granulosa cells and a basement membrane protect the ovum and prevent immune cell access during follicle development. Both the oocyte and its surrounding zona pellucida express immunogenic molecules foreign to the mother. Although neither the zona nor the ovum stimulates immunity in healthy women, autoimmune ovarian disease results from operational failure of the immune regulatory mechanisms required for normal tissue homeostasis.[59,60] Autoimmunity to oocyte antigens can be induced experimentally by treatment with antibodies to zona pellucida,[61] or by removal of the thymus shortly after birth, but this can be prevented by passive transfer of T lymphocytes from spleens of normal adult mice.[62] The protection-conferring population are now known to be Treg cells, which are actively educated to suppress immunity to zona-pellucida antigens within the lymph nodes draining the ovary.[63] Elegant experiments comparing male and female donors of Treg cells show the antigen-specific nature of the Treg suppressive activity and the necessity for the persistent presence of the cognate tissue antigen in generating the ovary antigen-specific Treg cells.[62]

A second, critical mechanism of protection of the ovary against autoimmune disease is deletion of ovary-reactive T cells undergoing development within the thymus. Failure of this mechanism occurs experimentally in mice with functional deletions of the autoimmune regulator (*Aire*) gene, and naturally in women suffering autoimmune polyendocrine syndrome type I (APS-I), in which the *AIRE* gene contains function-nullifying mutations.[64–66] Normally, this transcriptional regulator promotes expression of genes within the thymus, where T-cell development occurs, that are otherwise restricted to specific tissues.[64] Expression of these tissue-restricted antigens in the thymus ensures that immature self-reactive T cells, which arise frequently and at random during T-cell development, encounter the antigen and consequently are deleted.[67] Thus, in the absence of functional AIRE, self-reactive T cells targeting many endocrine organs, including the ovary, escape thymic elimination and erroneously leave the thymus. These deviant cells are then aberrantly activated upon encounter with the antigen in the periphery, even notwithstanding normal numbers and development of Treg cells. The fallout of AIRE deficiency is fulminant ovarian autoimmune disease, characterized by T-lymphocyte infiltration and production of autoantibodies targeting granulosa cells, luteal cells, and oocytes. Ultimately, this leads to complete loss of germ cells and infertility.[60]

Regulation of expression of the HLA antigens in oocytes may also be important. Oocytes lack the highly immunogenic class Ia (HLA-A, B) and class II (HLA-D) major histocompatibility (MHC) antigens,[68,69] and instead transcribe a gene in the class Ib family, HLA-G, whose protein products are not highly immunogenic[70] and instead elicit immunosuppressive and tolerogenic responses.[71] The unique features of the HLA antigens are discussed in greater detail below.

The Female Immune Response to Seminal Fluid at Coitus

The female response to paternal antigens begins at the time of insemination, after transmission of seminal fluid at coitus. Semen contains transplantation antigens, which as described in detail below, can be recognized by a host or recipient as foreign and elicit potent immune responses. MHC molecules are associated with sperm, seminal leukocytes, and/or desquamated genital tract epithelial cells, with a substantial concentration of the immunosuppressive MHC molecule, HLA-G5, within the extracellular fraction.[68] These and other seminal fluid antigens are the same as those that will later be expressed by the conceptus and gestational tissues if pregnancy ensues following coitus. Low frequency

of recombination within the MHC locus and preferential expression of paternal MHC expression in certain placental cells[72] increases the homology between paternal MHC antigens in the seminal fluid and conceptus tissue.

Seminal fluid contains signaling molecules that interact with estrogen-primed epithelial cells lining the female reproductive tract to activate expression of an array of cytokine and chemokine genes. This results in leukocyte recruitment and activation of innate and adaptive immune events in a sequence that resembles an inflammatory cascade.[73,74] This post-coital leukocytic response has been described in mice[75–77] as well as in pigs, rabbits, sheep, and several other mammals.[78–81] The female response to seminal fluid is thought to have a fundamental role in reproduction, because seminal fluid-signaling proteins linked with immunity and defense are present in such diverse organisms as flies, mosquitos, crickets, honeybees, rodents, and primates.[82]

Immune changes induced by seminal fluid facilitate preparation of the female reproductive tissues for pregnancy through clearance of debris and pathogens and providing an immune environment that ensures that surplus sperm do not elicit anti-sperm immunity. At least in some species, this pathway is also linked to activation of a state of active immune tolerance towards male transplantation antigens that will be shared after conception by the semi-allogeneic embryo.[73,74] The full effect of seminal fluid on female immune parameters is best characterized in the mouse, where within hours of mating, macrophages, dendritic cells, and granulocytes are recruited into the endometrial stroma and lumen.[77,83,84] Through a process of cross-presentation by female dendritic cells in lymph nodes draining the genital tract, seminal fluid antigens activate and expand inducible Treg cell populations.[26,85] These Treg cells subsequently circulate through the blood and boost the pool available for migration into the endometrium so as to assist in generating immune tolerance of the conceptus at implantation.[53]

The extent to which seminal fluid may elicit related effects on fertility is less well defined in women. After transmission at coitus, seminal fluid induces an inflammation-like response in the cervical tissue of women. This is characterized by extensive infiltration of macrophages, dendritic cells, and memory lymphocytes into the epithelial and deeper stromal tissues,[86] as well as a neutrophil exudate into the cervical canal.[87] This leukocyte infiltration is accompanied by elevated expression of *CSF2*, *IL6*, *IL8*, and *IL1A* as well as a wide array of other chemokine and cytokine genes.[86] The cervical response requires contact between seminal fluid and the female tissues, since condom-protected intercourse does not elicit comparable changes in gene expression and leukocyte recruitment.[86]

The inflammatory response to seminal fluid depends on seminal plasma factors originating in the seminal vesicle gland,[76] notably the potent immune-deviating cytokine transforming growth factor-β (TGFβ), which is synthesized in the latent form and activated in the female tract after ejaculation.[88] The TGFβ content of semen is extraordinarily high in all mammalian species so far studied, approximately five-fold that of serum, and comparable to colostrum, the most potent biological source of TGFβ known.[89] Similar data from rodents and primates indicates the likely conservation of this signaling pathway across mammalian species. Microarray and in vitro studies show that TGFβ is a critical male–female signaling agent in regulating the immune response to seminal fluid at coitus in women, but is not the only agent.[90] A second family of factors that interact with TGFβ in human seminal fluid signaling are the E-series prostaglandins, which are present in high concentrations in seminal plasma and mediate immune regulatory functions.[91,92]

A key role for seminal fluid TGFβ in inducing tolerogenic Treg cells after coitus is supported by experiments where exogenous TGFβ was found to boost vaginal Treg cell numbers and to alleviate fetal loss in the CBA/J × DBA/2J abortion-prone mouse model.[93] This potent immune-deviating molecule thus explains why seminal plasma is necessary for Treg-cell activation and promoting tolerance to paternal alloantigens at implantation.[26] However, the success of artificial reproduction technologies where pregnancy is initiated without female tract contact with seminal plasma suggests that while seminal fluid may contribute to promoting immune adaptation for pregnancy, it is not essential. Pregnancy is indeed possible after artificial insemination, or in vitro fertilization (IVF) and embryo transfer without seminal fluid exposure in rodents and livestock species, but the progression to pregnancy and fetal growth parameters in this situation can be compromised.[94] In women, lack of prior intercourse with the conceiving partner over several months prior to conception increases the likelihood of preeclampsia and fetal growth restriction,[95] and this may be linked to reduced abundance of Treg cells reactive with partner HLA antigens (see below).

Immunology of Preimplantation Embryo Development

Following ovulation and until fertilization, the ovum and its protective covering, the zona pellucida, traverse the fallopian tube (oviduct). Like the uterus, the oviduct is richly endowed with resident immune cells and secretes a remarkable array of cytokines.[96–98] The growth and development of the pre-implantation embryo as it traverses the female reproductive tract is influenced by the cytokines and growth factors secreted

into the luminal compartment from oviduct and uterine epithelial cells during the pre-implantation period. Embryos express cytokine receptors from conception until implantation; multiple cytokines exert different effects on cell number and viability, gene expression, and developmental competence.[99] The biological effects of several growth factors and cytokines targeting the pre-implantation embryo have been reviewed previously.[100–104] Experiments involving the addition of exogenous cytokines to embryo culture, neutralizing ligand or receptors, or using mice with null mutations in cytokine genes have shown that several factors including GM-CSF, CSF-1, LIF, heparin-binding epidermal growth factor (HB-EGF), insulin-like growth factor-I (IGF-I), and IGF-II promote blastocyst development, while others including TNF and IFNγ exert potent inhibitory effects. The exposure of embryos to the correct array of cytokines during development may be essential for later fetal and placental development and long-term health of offspring.[105]

In most women, there is no evidence for immune rejection of the ovulated egg or early cleavage-stage embryo. The pre-implantation embryo is potentially more susceptible to immune rejection than the oocyte because it expresses a wider array of immunogenic molecules, including "foreign" (paternal) allogeneic antigens. The assumption is that the zona pellucida and adherent granulosa cells provide protection and that T cells would not have ready access to the embryo until implantation. But since the HLA-A, -B, and -D antigens that would stimulate rejection remain unexpressed by the external trophoblast layer of embryonic cells,[68] the capacity of the maternal immune system to recognize the foreignness of the embryo would be limited regardless of the zona pellucida.

Nonetheless, clinicians report that 50% or more of potential pregnancies fail.[106] Inadequate development of the pre-implantation embryo, as well as insufficient receptivity of the endometrium, contribute to this high rate of implantation failure. Reproductive immunologists fully recognize the contributions of genetic disorders to these losses but postulate that some of the unexplained infertility might be due to failure of the fallopian tube to provide sufficient growth factor support for the pre-implantation embryo[104] or to inadequate immune protection mechanisms in the fallopian tube.[107,108]

Since cytokine secretion by the oviduct and uterus can reflect the presence of a range of inflammatory and nutritional stressors in the female, the cytokines released by the oviduct may provide signals that embryos sense and adapt to the pervading environment, or alternatively undergo demise due to the unfavorable conditions.[109] Additionally, dysregulated expression of oviductal cytokines combined with altered immune cell populations and impaired embryo-tubal transport has been implicated as an underlying cause of ectopic pregnancy.[98]

Another immunoevasive strategy is production of immune suppressive molecules by the pre-implantation embryo. For example, both oocytes and embryos transcribe and translate membrane-associated and soluble isoforms of HLA-G, respectively called HLA-G1 and HLA-G5.[69,110,111] HLA-G5 is present in the media of in vitro cultured embryos, and high levels signal capacity of the embryo to implant and develop successfully in patients undergoing assisted reproductive technologies.[69,112–114] Abundance of secreted HLA-G and the ultimate success of in vitro fertilization may also be related to HLA-G genotype.[112,115] Although HLA-G5 is known to exert profound immune cell suppression (reviewed by Hunt et al.[71]), its expression in the embryo could be related to an immune suppressive function, to facilitation of normal embryonic developmental processes, or both: embryos with HLA-G mRNA exhibit an increased rate of cleavage.

A further potential immune cell modulator secreted by the human embryo is human chorionic gonadotropin (hCG). This polypeptide hormone is released prior to implantation, although it is not usually present in sufficient amounts for identification in pregnancy detection kits at this early time. hCG binds to receptors on decidual macrophages[116] and acts to induce a tolerogenic phenotype in dendritic cells through induction of 2,3-indoleamine dioxygenase (IDO).[117] The effects of hCG are also elicited through induction of immune regulatory cytokines in endometrial cells, which exert direct effects on both developing embryos and the endometrial immune response.[118] Through direct effects on lymphocytes as well as cytokine-induced immune deviation exerted via tolerogenic dendritic cells, hCG suppresses the cytotoxic activity of T cells and instead promotes Treg-cell recruitment into the implantation site.[119]

IMMUNOLOGY OF EMBRYO IMPLANTATION

The Decidual Response

As the embryo attaches and then invades into the endometrium, progesterone levels surge and in response, dramatic changes take place in the local environment to accommodate and respond to the infiltrating conceptus-derived cells. A key event in the maternal reaction to embryo implantation is the decidual response of the endometrium. In women, this commences in the superficial endometrium in the latter half of the menstrual cycle under the influence of progesterone. In mice and several other species it occurs contemporaneously with, and in response to, embryo implantation into the

anti-mesometrial side of the uterus. Decidualization is a transformation in uterine stromal cell phenotype signals that involves complex interactions between ovarian steroid hormones, growth factors, and cytokines,[120,121] and potentially specific leukocyte populations including NK cells and dendritic cells.[33,34] Accompanying the decidual response is a dramatic change in the uterine extracellular matrix (ECM), which provides a structural framework for trophoblast invasion and supports an increase in vascular permeability and tissue edema.[120] In turn the decidual response alters resident leukocyte populations, as a consequence of the altered ECM affecting leukocyte adhesion, migration, and differentiation, acting in concert with direct effects of decidual transformation on chemokine synthesis.[50]

The Leukocyte Response to Implantation

The leukocytes present at the time of embryo implantation are specifically equipped: (1) to facilitate the decidual transformation; and (2) to deal with the initial challenge of invading embryo-derived trophoblast cells.[33] Under the influence of progesterone, the resident leukocytes become programmed by local cytokines and conceptus-derived signals into immune inhibitory profiles consistent with transition into the immune-privileged environment required to sustain semi-allogeneic pregnancy.[122,123] This pro-tolerogenic behavior in leukocytes is essential to protect and support the initial phase of placental morphogenesis when the genetically dissimilar, highly specialized cytotrophoblast cells from the implanted blastocyst advance directly into regions where these leukocytes reside (Figure 41.2).

Although the nonpregnant, cycling uterus is home to several subpopulations of leukocytes with the ability to initiate an antigen-specific immune response, major modifications are made in the uterine leukocyte populations concurrently with implantation to reduce the likelihood of anti-fetal immunity (reviewed in Refs 8, 9, and 124). The pregnant uterus is effectively reprogrammed from a mucosal site where Th1- and B-lymphocyte immunity is facilitated into a site where innate immune cells predominate and T-cell immunity is suppressed. Yet this early establishment phase when trophoblast cells first breach the epithelial surface of the uterus is most vulnerable to failure as a consequence of immune attack or insufficient endometrial support, with >50% of human embryos estimated to be lost at this time.[106] Antigen-specific immunity towards conceptus antigens, if present in the mother, could comprise a significant danger to ongoing development of the semi-allogeneic fetus.

The most prominent leukocyte subpopulations that persist in the peri-implantation uterus are NK cells and antigen-presenting cells that include macrophages and dendritic cells. B cells and many T cells disappear from the decidualizing endometrium,[23,125] leaving immune suppressive, anti-inflammatory regulatory T cells as the major remaining T-cell subset.[126] Treg cells are relatively enriched, whereas Th2 cells remain proportional

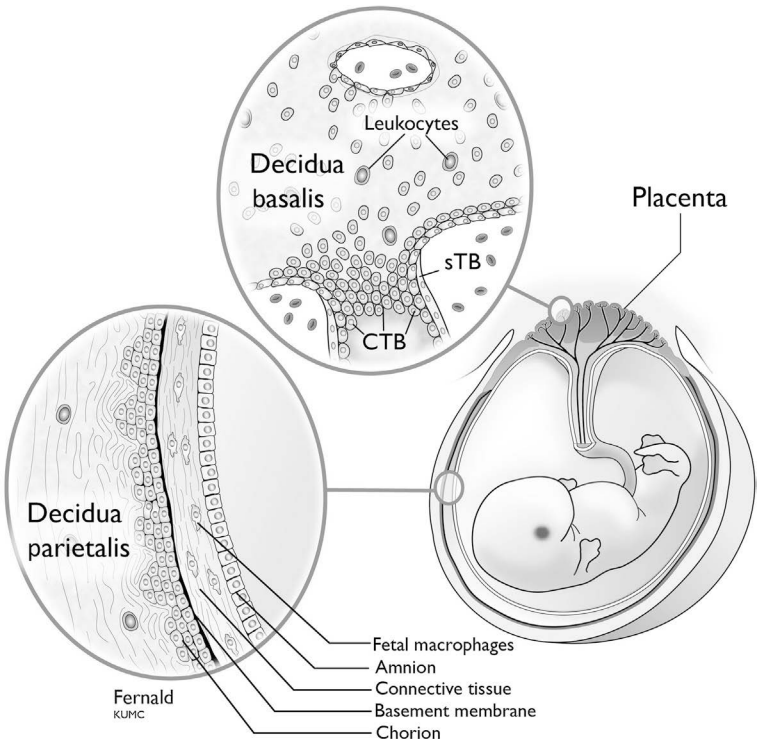


FIGURE 41.2 Anatomy of the maternal–fetal interface. The fetus is entirely surrounded and encased by placental and chorion membrane trophoblast cells. Maternal cells and fetal cells are intermixed in the decidua basalis, the site of attachment of the placenta to the uterus (upper insert) and between the chorion membrane and decidua parietalis (left insert). CTB, cytotrophoblast cells; sTB, syncytiotrophoblast.

to Th2 cells in the blood. Levels of Th1 and Th17 cells, on the other hand, are substantially diminished compared with levels in peripheral blood.[127,128] As a consequence of the cellular changes and their programming for anti-inflammatory rather than inflammatory responses, the pregnant uterus is a site where support of embryonic development rather than maintenance of host defense is favored. This is in contrast to the cycling uterus where defensins and other antimicrobials are abundant.[129] Because infections are less vigorously controlled in the pregnant than in the cycling uterus, a common consequence may be infection-associated preterm labor.[130]

Genetic models allowing depletion of specific leukocyte lineages show several leukocytes are essential for a normal decidual response and embryo implantation in mice. Although the data cannot be directly extrapolated to humans, the mouse models provide important leads for exploration of mechanisms and pathways in women. Dendritic cells,[34] macrophages,[58] and Treg cells[15] are all essential for implantation and progression of pregnancy. Dendritic cells and macrophages are essential regardless of fetal MHC disparity, consistent with critical roles in non-immune as well as immune-regulatory functions. When dendritic cells are depleted from *Cd11c-Dtr* mice, by administration of low-dose diphtheria toxin to transiently remove DT receptor-expressing $CD11c^+$ cells, formation of the decidua and ensuing placental development is terminated.[34] When macrophages are depleted from *Cd11b-Dtr* mice, where low-dose diphtheria toxin depletes DT receptor-expressing $CD11b^+$ cells, embryo development proceeds to the blastocyst stage but implantation fails. This is because macrophages are critical for the development of the vascular network within the corpus luteum that allows progesterone secretion and underpins endometrial receptivity.[58] Treg cells are absolutely essential for semi-allogeneic pregnancy but are less important for syngeneic pregnancy, underscoring their critical role for maternal tolerance of fetal MHC antigens.[15] Perhaps surprisingly, NK cells are not essential for pregnancy,[30] although in the absence of these cells decidual vasculature remodeling is compromised and placental development is impaired. The precise roles of these individual leukocytes are detailed in the section Immunology of Pregnancy: The Maternal Contribution later in the chapter.

GENETIC DISPARITY AND THE IMMUNOLOGICAL PARADOX OF PREGNANCY

The Fetal Semi-Allograft: Self–Nonself, and the Danger Theory

Following implantation, there is explosive growth of embryonic and extraembryonic tissues, the latter of which quickly begin to interdigitate with the maternal decidua in a manner that leads to juxtaposition between genetically disparate cells unparalleled in any other physiological situation. Because of the range of antigens expressed by the growing fetus and placenta that are foreign to the mother together with the expansive physical contact between the two, there is, conceivably, an immunological threat to the survival of the fetus even greater than that of the oocyte and pre-implantation embryo. These antigens include molecules derived from paternal genes as well as tissue-specific molecules that are unexpressed in other types of cells. Importantly, these antigens do not generally evoke an adverse immune response; in a healthy pregnancy, maternal cells do not attack fetal or placental cells, and antibodies in the maternal blood with specificity for fetal/placental surface antigens do not cause trophoblast death.

The "self–nonself" paradigm of transplantation immunity[2] has been helpful in shaping thought and informing experimental analysis of how the fetus, and particularly the placenta, evade maternal immune rejection in pregnancy. Transplantation theory would predict that foreign antigens expressed by the fetal-placental unit should act as "transplantation" antigens, which would stimulate graft rejection by recipient immune cells. In humans the major transplantation antigens are the HLA antigens, which are subdivided into class Ia (HLA-A, -B, and -C), class Ib (HLA-E, -F, and -G), and class II (HLA-D) (discussed in detail below). Consideration of the role of these antigens in transplant rejection led to discovery that a major adaptation that permits semi-allogeneic pregnancy is that placental trophoblast cells strictly regulate HLA gene expression and select for those that are least immunogenic (reviewed by Loke and King[131]). It seems clear that preventing maternal overreaction to fetal-placental antigens is important, since in addition to tightly controlled expression of HLA by trophoblast cells, the pregnant uterus and placenta contain an abundance of overlapping and complementary immunoprotective mechanisms. Although T lymphocytes generated in the thymus that bear TCRs specific for all foreign MHC are exceptionally numerous, in mice it seems cytotoxic T cells programmed for response to paternally inherited antigens are effectively barred from entering the decidua following implantation. A central mechanism by which this occurs was recently revealed using experimental murine models: epigenetic alterations preventing expression of essential T cell-recruiting chemokine genes take place in decidual cells, preventing entry of T cells even in the presence of strong systemic inflammatory signals.[132]

The "Danger Model" of immunity, originally put forward by Polly Matzinger,[133] has also informed thinking on the immune response to pregnancy. This model proposes that immune responses are not initiated by degree

of foreignness of an antigen to the host per se, but rather by its capacity to initiate tissue damage. When applied to pregnancy, the Danger Model would predict that because paternally inherited fetal antigens—and indeed any fetus, embryo, or oocyte antigens—constitute no risk to the mother, maternal T lymphocytes programmed to respond to these antigens should not be activated.[134] This is in contrast to immune responses generated against antigens associated with microbial pathogens, where conserved ligands or molecular motifs called pathogen-associated molecular patterns (PAMPs) (e.g., lipopolysaccharide (LPS) and viral nucleic acids) avidly bind to pattern recognition receptors (PRR). Pattern recognition receptors include Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors, and RIG-I-like receptors, and are expressed on leukocytes and many "nonprofessional" immune cells, including cells in the placenta (reviewed by Takeuchi et al.[135]). Ligand activation of PRR as a result of microbial infection stimulates a potent inflammatory response and promotes immunity. Upon necrotic or ischemic injury to tissues, endogenous damage-associated molecular patterns (DAMPs) can also bind to PRR and generate inflammation and immunity. With respect to normal pregnancy, it is likely that a lack of PRR ligands and thus failure to elicit "danger" signals reinforces other active and passive mechanisms by which immune rejection of the conceptus is avoided. Conversely, ischemic or necrotic damage to fetal-placental tissues in pathological pregnancies may promote inflammation as a result of DAMP/PRR signaling, further endangering pregnancy.[136]

Structural Features of the Human Maternal–Fetal Interface

The complex architecture of the gestational tissues and their anatomical and physical relationship with the mother is perhaps the single most crucial determinant of the immunological success of pregnancy. Most notably, the placenta and associated extraplacental membranes are principal players in promoting a peaceful immunological coexistence between the mother and fetus during pregnancy. Many types of placental cells participate in this arrangement, particularly the various populations of trophoblast cells. Trophoblast cells arise from the trophectoderm, the outermost layer of the blastocyst, and are normal occurrences only in pregnancy. On the whole, trophoblast cells comprise the single fetal cell type that interfaces with maternal blood and decidua (Figure 41.2). As such, these cells define the nutritional, endocrinological, and immunological interface between the mother and the fetus. These unique cells thus have a major impact on both local and systemic immunological processes in the mother, and this impact is tied to their anatomical positions within the placenta, their manner of self-renewal and turnover, and by their expression of paternally inherited alloantigens together with tightly regulated expression of immunologically relevant modulators.

Early Development of the Placenta and Extraplacental Membranes

Almost immediately upon implantation, the trophectoderm initiates a complex program of differentiation, ultimately giving rise to several structurally and functionally distinct trophoblast subpopulations. Archival tissue sections from human implantation sites suggest that trophectoderm-derived trophoblast cells promptly expand by proliferation, the outermost cells fusing to form a syncytium. From this point onwards, a "trophoblast shell" encases the inner cell mass, which concurrently initiates formation of embryonic and extraembryonic tissues. This basic anatomical arrangement between embryo and trophoblast persists for the duration of pregnancy: the embryo and fetus remain entirely sequestered from the mother by layers of trophectoderm-derived trophoblast cells (Figure 41.2).

After implantation, the precursors to all trophoblast cells in human placentas are the villous cytotrophoblast cells. Avascular chorionic villi separated by lacunae form and surround the embryo. By six weeks, the villi deepest within the uterine wall are invaded, as a result of chorioallantoic fusion, by extraembryonic mesoderm. Mesodermal and trophoblastic elements undergo branching morphogenesis to form mature, vascularized chorionic villi and the definitive placenta. Within the villi, cytotrophoblast cells proliferate and differentiate along one of two pathways. First, they can fuse to the outermost syncytiotrophoblast layer of the chorionic villi, effectively donating and replenishing fresh cytoplasm, macromolecules, and organelles. Alternatively, proliferating cytotrophoblast cells may break through the syncytiotrophoblast, exit the villus, and migrate into the decidua in formations of defined columns. These columns serve to physically anchor the placenta to the decidualized endometrium, which at its location beneath the placenta is called the decidua basalis. The migrating extravillous trophoblast emanating from the columns differentiate further, subdividing into those located in the decidual stroma (interstitial cytotrophoblast cells) and those lining the maternal spiral arteries (endovascular cytotrophoblast cells). The presence of endovascular cytotrophoblast cells signifies completion, at around 12 weeks of gestation, of the important transformation of the maternal endometrial spiral arteries from narrow, constricted vessels into broad conduits that supply the placenta with ample maternal blood at low pressure (reviewed by Burton and Jaunaiux[137]).

Whereas the deeper, vascularized chorionic villi progress to create the definitive placenta, the more superficial

villi, opposite the site of placental growth, are never invaded by mesoderm, and instead regress to form the chorion membrane. The chorion membrane is one stratum of four, collectively called the extraplacental membranes. From the fetal to maternal aspect, the membranes are comprised of amnion, which encompasses the amniotic cavity wherein the fetus resides and develops; two connective tissue layers; chorion; and maternal decidua capsularis (Figure 41.2). The mesodermally derived connective tissue between the amnion and chorion is replete with fetal macrophages. Atop the chorion lies the decidua capsularis, which, as the fetus and amniotic fluid expand and obstruct the uterine lumen, becomes progressively thinner and eventually fuses to the decidua parietalis. While the placenta grows to occupy up to one-third of the perimeter of the maternal–fetal interface, the extraplacental membranes constitute the remaining area. Together, these structures comprise a chimeric unit of two intact but genetically distinct entities, an arrangement that in physiology is entirely unique to pregnancy.

Syncytial Shedding: Knots, Microvesicles, and Exosomes

As well as whole cells, fragments and vesicles that range from nanometer scale to large multinucleated syncytial knots are shed into the maternal circulation (Figure 41.3) (reviewed by Redman and Sargent[138]). The smaller vesicles have been classified as plasma membrane-derived microvesicles. These bleb from the cell surface and measure up to 1 μm in diameter. Additionally, exosomes, which are 50–150 nm, are derived intracellularly from multivesicular bodies. Syncytial knots may represent collections of effete or apoptotic nuclei that arise as a consequence of physiological regeneration of the syncytium: hypothetically, as villous cytotrophoblast cells fuse and replenish the syncytium, effete nuclei cluster and are released into the maternal blood together with aged cytoplasm and other organelles.[139] Although the origin and function of syncytial knots is controversial,[140–142] it is clear that an abundance of fetal material accesses the maternal blood during pregnancy.

Further, placentally derived vesicles, small and large, may transport fetal antigens and/or immunomodulatory proteins to maternal organs. Where the shed material ultimately alights likely depends, at least in part, on their size. Knots, which measure up to 100 μm, can become trapped within the first capillary bed they encounter upon exiting the uterine vein, which is in the lung.[143,144] Small sized microvesicles can pass through the capillary beds and readily circulate within maternal blood.

Although it is important to note that the majority of microparticles in maternal blood originate from maternal platelets,[138] the placental contribution of exosomes and microvesicles undoubtedly changes the maternal immunological milieu. Circulating placental exosomes, identified by their expression of placental alkaline phosphatase, are found in maternal blood and contain biologically active components including Fas ligand and MHC class II antigens that can influence expression of T-cell activation markers.[145] Chorionic villous explant-derived exosomes generated in vitro similarly retain immunosuppressive proteins possessed by their syncytiotrophoblast and villous cytotrophoblast parents. Among these proteins are the HLA and B7 family proteins, syncytin-1, and biologically active NKG2D ligands.[146–148] These features may be shared by extravillous trophoblast cells, as these cells too appear to be high producers of exosomes that can influence macrophage function and differentiation.[149–151]

In addition to exosomes, larger microvesicles have been shown to elicit inflammation by maternal endothelial and immune cells.[138] Further, both microvesicles and exosomes have important implications for the adaptive immune response to fetal antigens in pregnancy. This material can impact maternal endothelial cells to influence their activation status and cytokine expression, and are likely also to be taken up and processed by maternal antigen-presenting cells in peripheral lymphoid organs. Depending on whether the originating trophoblasts are necrotic or apoptotic, either tolerogenic or immunogenic responses could be elicited.[138,152] Thus in vivo, similar placental immunomodulatory pathways could influence both the innate and adaptive maternal immune response to pregnancy.

Maternal–Fetal Cell Coexistence and Exchange

In species such as humans, rodents, and nonhuman primates where placentation is hemochorial, fetal cells invade deeply into maternal tissue and are in direct contact with maternal cells, where they would be expected to be at considerable risk of immunological attack. The most intimate associations between these genetically disparate cells are in the decidual stroma, where fetal cells intermingle with maternal cells, first in the maternal spiral arteries undergoing remodeling, and then in the chorionic villi, which are awash with maternal blood from the end of the first trimester onwards. At each of these sites, interstitial, endovascular, and chorionic cytotrophoblast cells neighbor maternal decidual, endothelial, and blood cells and readily coexist without evidence of inflammation. Ultimately, most of the vascular endothelial cells proximal to the placenta are entirely replaced by the endovascular trophoblast cells, and all syncytiotrophoblast cells are bathed in maternal blood. Thus, each trophoblast population has developed strategies for advancing and differentiating within the potentially immunologically hostile maternal tissue, while

demonstrating complete resistance to cytotoxic cells and other potentially destructive immune mediators circulating in maternal blood and tissues.

Beyond the maternal–fetal interface, maternal cells remain mainly in maternal tissues and fetal cells in fetal tissues. This is not an absolute separation, however, and there is compelling evidence that fetal cells traffic into peripheral maternal tissues, and maternal cells into peripheral fetal tissues (Figure 41.3). Termed fetal and maternal microchimerism, respectively, a proportion of these microchimeric cells survive following pregnancy and can reside alongside their familial host cells for substantial periods—even decades—influencing one another in unexpected ways.

That fetal microchimeric cells survive in the mother is likely due to several factors, including a failure to express a sufficient density of the MHC antigens to trigger graft rejection, a lack of "danger" signals, and active tolerogenic mechanisms. The origins and functions of the itinerant cells remain obscure, but both beneficial and detrimental effects on maternal health have been proposed. For instance, fetal microchimerism may stimulate maternal scleroderma, a graft-versus-host disease-like autoimmune disorder, and/or contribute to altered risk of rheumatoid arthritis.[153–155] Whether or not, and how, the microchimeric cells influence maternal autoimmune disease most likely depends on the genetic relationship between mother and fetus: both HLA genes and potential autoantigens are likely to be important (reviewed by

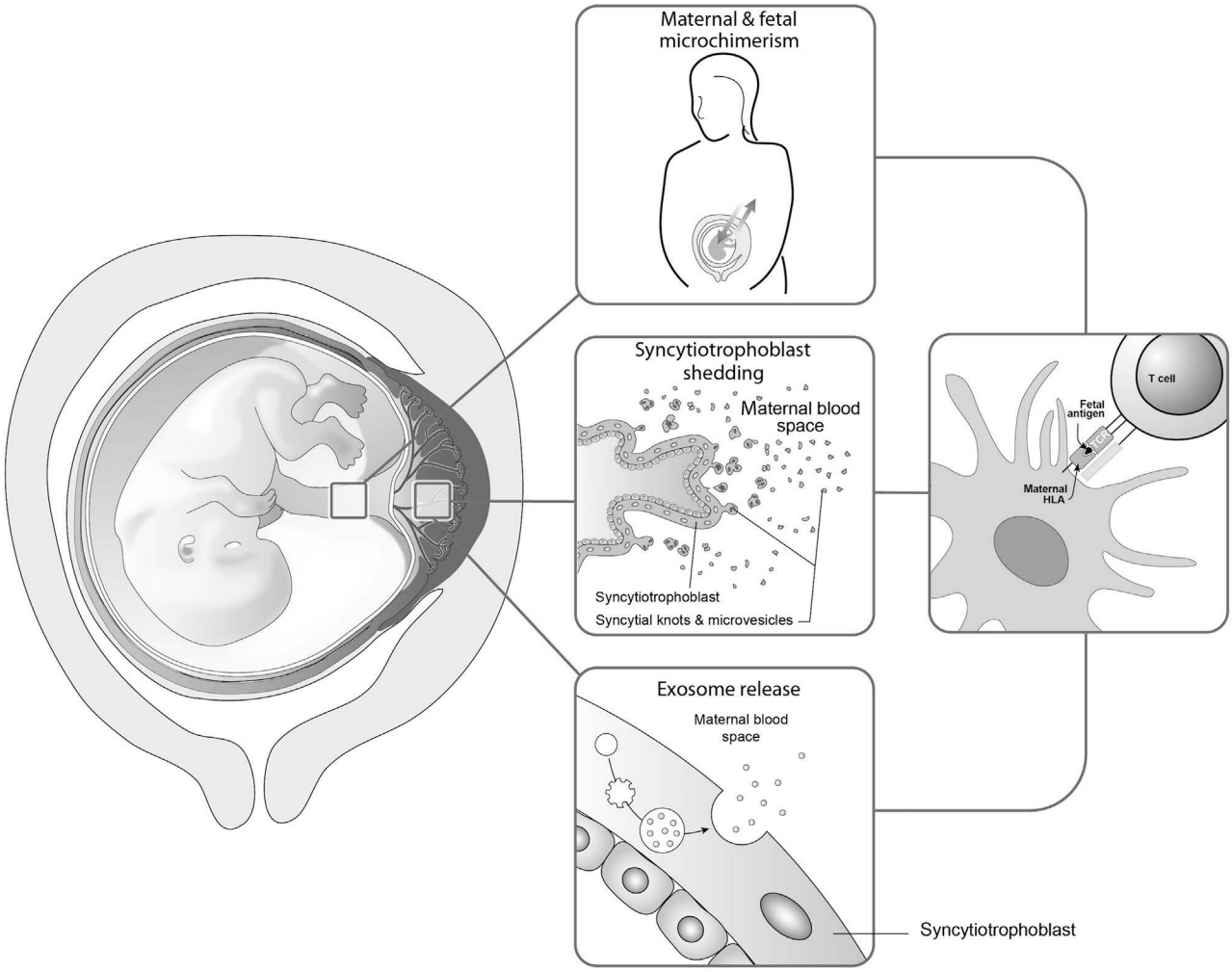


FIGURE 41.3 Microchimerism and exchange of fetal–maternal material across the placental barrier. A small number of fetal cells circulate systemically in the maternal blood, and conversely, maternal cells are present in the fetal circulation (upper insert). In addition to intact cells, substantial amounts of subcellular material are shed from placental syncytiotrophoblasts in the form of fragments ranging in size from large multinucleated syncytial knots and microvesicles (middle insert) to nanoscale-sized exosomes (lower insert). This shed material is thought to provide antigens that can be presented by maternal antigen-presenting cells and other immune-regulatory signals that influence the maternal immune response in a systemic manner (right insert). *Source: Reprinted from Placenta 32 (Supplement B, Trophoblast Research vol. 25), M. Petroff: Fetal antigens: identity, origins and influences on the maternal immune system, S176-S181, Copyright 2011, with permission from Elsevier.*

Nelson[155]). Additionally, the fetal cells might comprise a reservoir of stem cells for organ regeneration and repair and/or participate in sustaining long-term tolerance in the mother to fetal antigens.[156–158]

In humans[159] and in mice,[160] maternal microchimerism also occurs: small populations of maternal cells are found in fetal tissues, and these persist after birth and into adult life. Understanding resistance of maternal cells to fetal attack is less paradoxical than the reverse situation; indeed it seems unsurprising that maternal cells remain unharmed in the fetus, as the fetal immune system is still undergoing development: antigens encountered by the fetal/neonatal immune system are recognized as "self" and are tolerated. Furthermore, there is more recent evidence in humans that maternal cells traffic across the placenta and access the fetal lymph nodes, resulting in induction of Treg cells that promote fetal tolerance to maternal antigens.[161] Thus, it is likely that both centrally (in the thymus) and peripherally (in the lymph nodes), fetuses develop potent, perhaps lifelong tolerance to non-inherited maternal antigens (NIMAs) due to maternal microchimerism.

Maternal Awareness of Fetal Antigens

Despite the altered antigenicity and lack of "danger" stimuli associated with pregnancy, it is now well established that the adaptive immune compartment is also engaged as part of the normal maternal immune response to pregnancy. It has been known for decades that some pregnant women make antibodies towards fetal HLA antigens—indeed, serum from pregnant women is a rich source of anti-HLA immunoglobulin originally used for HLA typing.[10,70] More recently, it has become clear that activated T cells reactive with fetal HLA are detectable in the peripheral blood[162] and decidua of pregnant women.[11] Importantly, the presence of antibodies and immune cells appears entirely consistent with healthy pregnancy and birth, and likely occurs to some extent in most women.[11,162,163] This shows that maternal lymphocytes are not ignorant of the embryo, but instead are actively primed to recognize conceptus antigens including paternal MHC.

Strong evidence for maternal immune awareness of fetal antigens is evident in mice, where T-cell transgenic models have been very informative.[13,14] In these model systems, which are now commonly used to study the adaptive immune response to pregnancy, endogenous or transferred T cells have been clearly shown to interact with natural or genetically introduced conceptus antigens.[13] In healthy pregnancy, the T-cell response ranges from activation and proliferation to anergy and deletion, but when T cells are inappropriately activated, rejection of the fetus in an alloantigen-dependent manner can occur.[15]

Together, these considerations underscore the complexity of the issues at play in the maternal immune response to pregnancy. The consensus view at the time of writing is that despite several strategies to diminish the antigenicity and immunogenicity of placental tissue in contact with maternal blood, ultimately an adaptive immune response to pregnancy is detectable. In the last 5 to 10 years, this has refocused research on the question of how maternal immune awareness in the adaptive compartment can be reconciled with pregnancy success. In outbred animals such as humans, it may be that no one individual strategy is an absolute requirement and that considerable redundancy exists between multiple overlapping mechanisms of suppression and tolerance. The following sections will discuss the range of fetal and maternal strategies that are believed to contribute.

IMMUNOLOGY OF PREGNANCY: THE MATERNAL CONTRIBUTION

During early placental development, a range of adaptations in the local and systemic maternal immune environment contribute to accommodating the invading conceptus tissues for the duration of pregnancy. This permissive response to pregnancy is driven initially by progesterone released by the corpus luteum and then by progesterone and other factors released from the placental trophoblast cells (see below for details). Progesterone and placental hormones sustain a specific local cytokine profile, and these soluble factors combine to support specific immune cell phenotypes in the decidua. These cytokine and leukocyte parameters conspire to suppress inflammation and generate a mother's functional immune tolerance of conceptus tissues, which persists from implantation until late gestation when parturition commences. As well as regulating the immune response, immune cells at the site of maternal contact with conceptus-derived placental trophoblasts participate in the tissue remodeling and particularly the changes to the maternal vasculature that enable adequate placental access to the maternal blood supply required to support optimal fetal development.

Immunological features of the pregnant uterus have been extensively reviewed.[9,164,165] The change of leukocyte subpopulations such that cells of the innate immune system predominate and T-cell cytotoxicity is diminished, is of critical importance. Although small numbers of T lymphocytes are present throughout gestation, these are mainly Treg cells. NK cells and mononuclear phagocytes are the predominant subgroups of immune cells in the pregnant uterus.

The maternal portion of this interface depends on a signaling circuit wherein the hormone progesterone constitutes the major force in reconstructing the uterus

into an anti-inflammatory and immune-privileged site. Progesterone interferes with the NFκB pathway to inhibit production of pro-inflammatory molecules,[166] and together with the elevated estrogens characteristic of pregnancy drives cells of the innate immune system into suppressive profiles characterized by diminished production of inflammation-associated cytokines.[167,168] Suppressed NFκB reduces expression of the Th1 transcription factor T-bet,[166] which likely explains the profound effect of progesterone in skewing T lymphocytes towards production of IL-10 and IL-4.[169] Thus, macrophages, NK cells, and the few T lymphocytes remaining in the decidua exhibit anti-inflammatory patterns of cytokine secretion under the direction of this powerful hormone influence.

Additionally there are systemic consequences for pathogenesis of various disease states, due to the altered immune environment in pregnancy. The reduction in activity of pro-inflammatory cytokines and natural killer cells, inflammatory macrophages, and helper T-cell type 1 (Th1) cells, combined with elevated Treg cell activity and production of anti-inflammatory cytokines, reduces the severity of diseases caused by inflammatory responses (such as arthritis and multiple sclerosis) and increases the severity of diseases that are mitigated by inflammatory responses (such as influenza and malaria).[167]

The Cytokine Response to Pregnancy

Following the initial uterine inflammatory response in the peri-conceptional phase, progesterone-induced anti-inflammatory cytokines such as TGFβ, IL-10, CSF1, and LIF gain prominence. Their synthesis in luminal and glandular epithelial cells drives nearby maternal immune cells into an immunosuppressive mode. Although the pregnant uterus produces several cytokines with immunosuppressive properties, IL-10 and TGFβ have been identified as of critical importance from an immune perspective.[170,171] The prominence of IL-10 synthesized by macrophages as well as epithelial cells was first shown in pregnant mice[5] and is a feature of the human decidua as well.[172] This cytokine not only directs T lymphocytes away from cytotoxic activity but also elicits macrophage production of HLA-G[173,174] in a feed-forward pathway that culminates in their release of the key immune suppressive cytokine, TGFβ.[175]

In mice, endogenous TGFβ is supplemented by TGFβ delivered in the male seminal fluid[176]; in rats, the cytokine is synthesized in diverse uterine cells[177]; and in women, TGFβ may be produced in uterine macrophages as a consequence of encounter with placental HLA-G5.[175] The TGFβ, together with IL-10, serves to induce specific phenotypes in local leukocytes that include alternatively activated M2 macrophages and tolerogenic dendritic cells. These ultimately prevent activation of cytotoxic immunity in the uterus. In this effort, the uterine cytokines are critically aided by direct effects of progesterone and prostaglandin E_2 (PGE_2), both of which target leukocytes directly and are highly anti-inflammatory and immunosuppressive.[168,178,179]

Studies in cytokine-null mutant mice enable the specific functions of individual cytokines to be discriminated from those with redundant roles, and the specific function of individual cytokines to be defined. Notably this strategy has identified a critical role for LIF in inducing maternal endometrial receptivity, particularly the decidual response that is essential for embryo implantation.[180] IL-11, which is in the same cytokine family as LIF and signals through related gp130-associated receptors, is also critical for the decidual response and implantation success.[181] The cytokine CSF1 was the first identified as being required for normal fertility.[182] Despite its function as a regulator of uterine macrophages, infertility is mainly the consequence of CSF-1 effects on normal ovulation and estrous cycle progression.[183] In contrast, other cytokines such as IL-10 are dispensable for pregnancy unless an inflammatory insult is administered, when mice deficient in this cytokine experience substantially elevated susceptibility to miscarriage,[184] which is at least partly due to altered NK cell activity.[185] Each of these same cytokines are synthesized in response to progesterone in the decidual tissues of early human pregnancy and has been implicated as contributing to the immune adaptation sustaining pregnancy.[186]

The Innate Immune Response to Pregnancy

Some of the most profound changes in pregnancy are reflected in local and systemic populations of leukocytes classified as comprising the innate immune response. The individual subsets of key innate cell populations are considered individually below.

Uterine NK Cells

Uterine NK cells (uNK cells) are the most prominent leukocyte in the early human decidua.[187] Compared with NK cells in the blood, the majority of uNK cells in the decidua are noteworthy for their unusual structural and functional characteristics and attenuated surface marker expression.[6,188] In women these cells have a defined chronological profile where the cells are numerous during the first two trimesters, comprising 20–40% of the decidual stromal cells, and then decline to undetectable levels as parturition approaches (reviewed in Refs 6 and 21). Human uNK cells are small cells of the lymphoid lineage that contain distinct granules and express a pattern of cell surface markers, $CD56^{bright}CD16^{-}$, which distinguishes them from NK cells in the blood, most of which are $CD56^{dim}CD16^{+}$. In the mouse and rat, the uNK cells are readily identified throughout pregnancy by their exceptionally large size and abundant granules.[188] The life-cycle

and cytokine regulation of the mouse uNK cell has been extensively documented, and includes degranulation in the last trimester and replenishment from the spleen in an IL-15-dependent manner (reviewed in Ref. 30).

NK cells are traditionally known for their surveillance function; in most mucosal sites, NK cells recognize and kill infected and abnormal cells. Unlike other mucosal NK cells, uNK cells do not readily kill target cells despite having reservoirs of killer molecules such as granzymes and TNF in their granules.[189] Failure to readily exhibit killing function is in part their response to decidual cell-derived signals that include CCL2, which induces suppressor of cytokine synthesis-1 (SOCS1) and suppresses perforin synthesis.[190] The cytokine profiles of the uNK cells are generally consistent with their functional differences to blood NK cells. These produce both Th1-type cytokines such as IFNγ and IL-18 that are associated with inflammation and cell activation, and Th2-type cytokines such as IL-10.[191]

NK cells recognize and destroy aberrant host cells on the basis of host cell failure to exhibit normally expressed HLA, or expression of NKG2D receptors such as MHC class I polypeptide-related sequence-A and -B (MICA/MICB).[192] Avoiding NK cell lysis may be one reason why cytotrophoblast cells migrating into the uNK-replete decidua exhibit cell surface MHC class I antigens. The HLA-C, HLA-E, and HLA-G molecules expressed by human trophoblasts are likely to have intrinsic protective capacity. In particular, interactions between trophoblast cell HLA-E and uNK CD94/NKG2 inhibitory receptors cause loss of killer function in human uNK cells.[193] Although HLA-G can modulate immune responses in blood NK cells, decidual NK cells are less responsive.[194] HLA-C is also present on extravillous trophoblast cells, and this polymorphic HLA class Ia molecule appears particularly important for interactions with uNK killer cell immunoglobulin-like (KIR) receptors, as explained in detail below.[195]

The general principle that uNK activities are beneficial to pregnancy is well accepted, mainly as a consequence of studies in genetically modified mice, where a range of elegant genetic strategies have been utilized to define their roles. Deficiency of uNK cells reveals a key role for these cells in modification of the maternal vasculature via production of IFNγ,[196] which in turn promotes placental growth and development.[197] Additionally these cells have pro-angiogenic functions in generating the more extensive decidual vasculature that accompanies placental development.[198] However, despite considerable experimentation, the roles these cells play in human pregnancy remain unclear.[199] In particular, the possibility that uNK cells contribute to "quality control" of the implantation process[200] requires further investigation. This might be achieved through their influence on commitment to decidualization versus menstruation,[33] or NKG2D-mediated elimination of conceptus tissue under nonconducive circumstances such as viral infection.[185,201] The commitment to exert cell-mediated killing activity against trophoblasts is also influenced by macrophage, dendritic cell, and Treg cell-derived signals, principally involving modulation of suppressive TGFβ and TLR-derived danger signals.[202,203]

Decidual Macrophages

Macrophages are a consistent feature of the decidua throughout the course of pregnancy. They average 15–20% of the decidual stromal cells in women,[124,204] where they are frequently located close to invading cytotrophoblast cells, adjacent to the uterine glandular epithelium, and proximal to uterine blood vessels. As with macrophages in other locations, these cells are highly versatile and multifunctional, with extraordinary capacity to alter their phenotype and behavior in response to environmental signals. They are thought to be critical to host defense, uterine tissue remodeling and homeostasis, and local immune modulation (reviewed in Refs 122, 197, and 205).

In human decidua, the cells are ubiquitous and make valuable contributors to defense, being fully capable of phagocytosis and destruction of microorganisms. In mice, macrophages are less abundant in the decidua, and this is believed to contribute to loss of resistance to infections such as *Listeria monocytogenes* in pregnancy.[206] The human decidual macrophages are activated, as evidenced by their expression of HLA class II, CD11c, and CD86 antigens (reviewed in Ref. 71), and therefore appear capable of presenting microbial or other exogenous antigens to T lymphocytes. Yet decidual macrophage cytokine profiles indicate that the cells are programmed into an immune suppressive, alternatively activated (so-called M2) mode, producing powerful immunoinhibitory cytokines such as IL-10 and TGFβ1,[172,175] presumably in response to M2-inducing TGFβ and PGE_2 synthesized in other endometrial cells.[207] In the mouse, macrophages themselves are a source of PGE_2,[179] a molecule with profound immunosuppressive properties, but human decidual PGE_2 appears to arise from endometrial stromal cells that have differentiated into decidual cells.[206] Decidual macrophages are reported to express B7-H1,[208,209] an inhibitory member of the B7 family of costimulatory molecules, indoleamine dioxygenase (IDO, as well as ILT-3, DC-SIGN, and MS-1 (reviewed in Refs 7, 11, and 97)), all of which are markers associated with immune modulation characteristic of an M2 suppressive profile.

Like M2 macrophages involved in fetal development, decidual macrophages are believed to contribute to trophoblast invasion and placental development through their secretion and regulation of a range of matrix metalloproteinases and other proteases, growth factors, chemotactic

molecules, cytokines, and a range of matrix components. These agents, the precise patterns of which are fine-tuned by local microenvironmental signals, afford macrophages key roles in the tissue restructuring and angiogenesis that are central to placental development.[205] Decidual macrophages are also highly competent in the induction and effective clearance of apoptotic cells, which in the uterus would be necessary to accommodate the expanding fetus as pregnancy progresses.[210]

Under certain conditions such as intrauterine infection or preeclampsia, the environmental factors that support alternatively activated decidual macrophages are overcome by TLR-mediated danger signals and the cells instead assume a pro-inflammatory profile.[205,211] In preeclampsia, decidual macrophages produce TNF, which has toxic effects on migrating trophoblast cells and might account for the diminished numbers of viable cytotrophoblast cells in the preeclamptic decidua.[212] In gram-negative infections, the decidual macrophages produce extraordinarily high levels of TNF, other inflammatory cytokines, and PGE_2, which ultimately induce preterm labor (reviewed in Refs 213–215).

Decidual Dendritic Cells

The decidua contains another type of innate immune cell, the dendritic cell (reviewed in Refs 216 and 217). The principal function of dendritic cells is to regulate the adaptive immune response through a specialized capacity to take up, process, and present antigens to T lymphocytes, and to govern the activation response of T cells through powerful immune-regulatory activity. Although fewer in number than macrophages, dendritic cells are ontologically related; a large proportion of dendritic cells in the uterus exhibits characteristics consistent with a myeloid origin. Decidual dendritic cells fall into two broad categories: $CD83^+$ mature dendritic cells, which comprise approximately 1% of decidual stromal cells,[218] and $CD83^+$ immature macrophage/dendritic cells.

Like decidual macrophages, mature $CD83^+$ decidual dendritic cells exhibit an immunosuppressive phenotype and features of so-called "tolerogenic dendritic cells".[219] Tolerogenic dendritic cells control the activation and expansion of Treg cells and are crucial for maintenance of immune tolerance and prevention of autoimmune disease.[220] This subset is characterized by attenuated expression of co-stimulatory molecules CD80 and CD86, and absence of the Th1-inducing cytokine IL-12.[220] Expression of IDO is another defining feature of tolerogenic dendritic cells. IDO expression allows dendritic cells to directly activate resting Treg cells for potent suppressor activity, and appears to be crucial for converting $CD4^+CD25^+FOXP3^+$ Treg cells from $CD4^+CD25^-$ T cells and promoting their suppressive activity.[221]

Various immature dendritic cells populations are present in the decidua, including a unique DC-$SIGN^+$ $CD14^+CD83^-$ population that exhibits high proliferative activity and close physical association with uNK cells.[222] These cells fail to stimulate resting T cells and preferentially activate inducible Treg cells.[223] The DC-$SIGN^+$ cells switch their phenotype in response to inflammatory cytokines, differentiating into mature $CD83^+$ dendritic cells capable of activating T-cell immunity.[222] DC-SIGN is utilized for immune evasion by several viral and bacterial pathogens, and binding alters both cytokine production and antigen presentation in ways that benefit the pathogen.[224] Thus, DC-$SIGN^+$ cells are implicated as central players in the development of the pregnant uterus as an immune privileged site benefiting the fetus.

Certain decidual cytokines, including TGFβ, IL-10, GM-CSF, granulocyte-CSF (G-CSF), and IL-4, are linked to differentiation of tolerogenic dendritic cells that consistently induce $CD4^+CD25^+$ Treg cells.[225] The glycan-binding protein galectin-1 is an additional key regulator of tolerogenic dendritic cells capable of inducing Treg cells.[226] HLA-G also directs induction of tolerogenic dendritic cells, acting by ligating the LILRB2 receptor to disrupt the HLA class II–mediated antigen presentation pathway.[227] These tolerogenic properties of HLA-G are linked with Treg cell induction and in part are mediated through endogenous HLA-G synthesis within decidual DC-SIGN+ dendritic cells.[223]

Recent studies indicate that uNK cells have an instrumental role in controlling the tolerogenic phenotype of decidual dendritic cells. In mice, acute NK ablation using a diphtheria toxin-mediated transgenic approach switches dendritic cells to an immunogenic function.[228] As in mice, human decidual dendritic cells interact closely with NK cells,[229] but the relevant NK-cell derived signals are yet to be identified.

The Adaptive Immune Response to Pregnancy

In the immune system, T lymphocytes can be broadly categorized into CD8+ cytotoxic T cells (CTLs) and CD4+ T helper cells (Th cells). Th cells can be further stratified into Th1 cells, which produce inflammatory cytokines; Th2 cells, which produce anti-inflammatory cytokines and promote B lymphocyte development; Th17 cells, which are pro-inflammatory and affect antipathogen immunity and graft rejection; and Treg cells, which are anti-inflammatory and immune-suppressive (Figure 41.4). While differentiation of Th1 and Th2 cells is generally viewed as irreversible, the more recently described Th17 and Treg cells are less stable and there is greater plasticity for movement between these two phenotypes when environmental conditions change.[230]

At a systemic level, cytotoxic T lymphocytes generated in the thymus that bear TcR specific for foreign MHC are

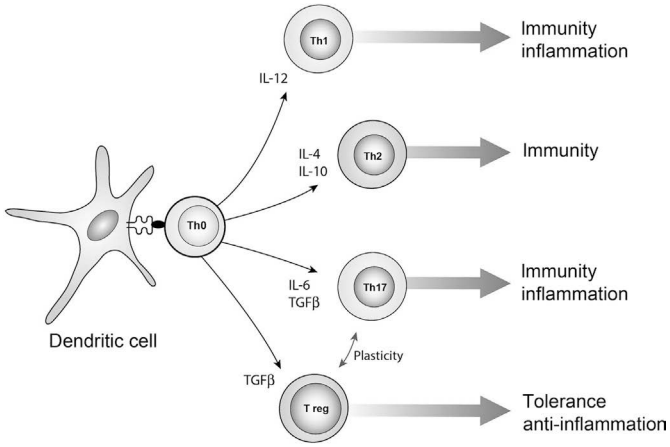


FIGURE 41.4 Funtional differentiation of Treg cells. The microenvironmental context in which naive CD4+ Th0 cells encounter their cognate antigen is a principal determinant of their differentiation fate and development into Treg cells as opposed to Th1, Th2, or Th17 cells. Treg cells confer immune tolerance and suppress inflammation while Th1, Th2, and Th17 cells mediate protective immunity and are linked with inflammation. Signals originating from the dendritic cell presenting antigen to the Th0 cell, as well as the relative concentrations of key cytokines in the immediate vicinity, are instrumental. IL = interleukin; Th1, Th2, Th17 = T-helper type 1, type 2, and interleukin 17-producing Th cell; TGF = transforming growth factor.

exceptionally numerous, but T cells programmed to kill cells bearing paternal MHC antigens are essentially eliminated from the decidua following implantation. Various theories have been advanced to explain the mechanisms by which this local skewing in T-cell phenotype occurs. After discovery of the first division in Th cell phenotype between Th1 and Th2 phenotype in the 1980s, Wegmann and co-workers put forward the proposal that pregnancy could be considered a Th2-type phenomenon.[5] In normal pregnancies, Th2 cytokine levels are high at the maternal–fetal interface, and antibody responses in the mother are strong. Cells of the innate immune system residing in the pregnant uterus and their Th2-type cytokines undoubtedly contribute to this state, but a substantial body of evidence indicates that placental cells are the primary source of the immune-deviating cytokines that program T cells, principally through their actions on the dendritic cells and macrophages that induce and control T-cell phenotypes.

Although appealing in its simplicity, the concept of a Th2 bias in pregnancy is an oversimplification, and it is clear that many Th1-type cytokines, such as IFNγ and TNF, have important roles in homeostasis of the pregnant uterus and placenta.[231] Indeed it now seems the key point is that detrimental Th1 and Th17 cells are suppressed, rather than Th2 cells promoted. Th1 and Th17 cell-mediated responses are dampened by placental anti-inflammatory cytokines such as TGFβ and IL-10 as well as progesterone and prostaglandins, whereas Th2-regulated antibody responses may be maintained and even elevated by two nonapoptosis-inducing TNF superfamily ligands produced in placentas, BAFF (also known as BlyS [B lymphocyte stimulator], TALL-1, THANK, zTNF4) and APRIL (a proliferation-inducing ligand)[232] (Figure 41.5).

Infections can overcome the immunosuppressive environment at both the local and systemic levels, with generation of high levels of Th1-type responses.[211,213,215] This defense against pathogens may be a lifesaving response for the mother, but devastating effects can be incurred by the fetus, with the pro-inflammatory shift to Th1 and Th17 responses identified as a key trigger for preterm labor.[233] The major cellular element in the maternal decidua T-cell population responsible for suppressing Th1 and Th17 cells under normal circumstances is Treg cells.

T Cells

In women, T lymphocytes comprise about 10% of the leukocytes in the decidual tissue of early pregnancy, and the majority of these are CD8+ with a relatively smaller population of CD4+ helper T lymphocytes. The precise phenotypes and specific cytokine expression profiles of decidual T cells have been explored, and considerable heterogeneity is evident.[234,235] Their antigenic specificities also remain unknown, but the majority of those expressing γ/δ TCRs incorporate gene segments that indicate potential recognition of a wide array of self and/or trophoblast antigens.[236,237] Activated T cells reactive with fetal HLA are detectable in the peripheral blood[162] and decidua of pregnant women.[11] A high degree of MHC mismatch between maternal and fetal HLA increases the numbers of these cells.[11,162,163] Experiments using reagents to evaluate the reactivity of TCRs expressed by T cells shows that at least half of all pregnant women have T cells with specificity for fetal epitopes, and these retain a functional memory phenotype throughout pregnancy without causing harm to the fetus.[238]

A similar scenario is evident in mice, with T lymphocytes increasing substantially in pregnancy such that α/β and γ/δ TCR+ T cells comprise up to 20% of total lymphocytes in midgestation decidua.[239] Specificity for paternal class I MHC antigens is detected among CD8+ T cells in lymph nodes draining the uterus, although evidence of downregulated TCR expression suggests functional unresponsiveness in these cells.[14]

To some extent, T-cell responses to alloantigens in pregnancy may have benefits for pregnancy outcome. For example, mice mated with MHC-disparate males have greater numbers of fetuses than inbred mice, and the fetuses are heavier, potentially due to greater placental access to maternal nutrients from increased dilation

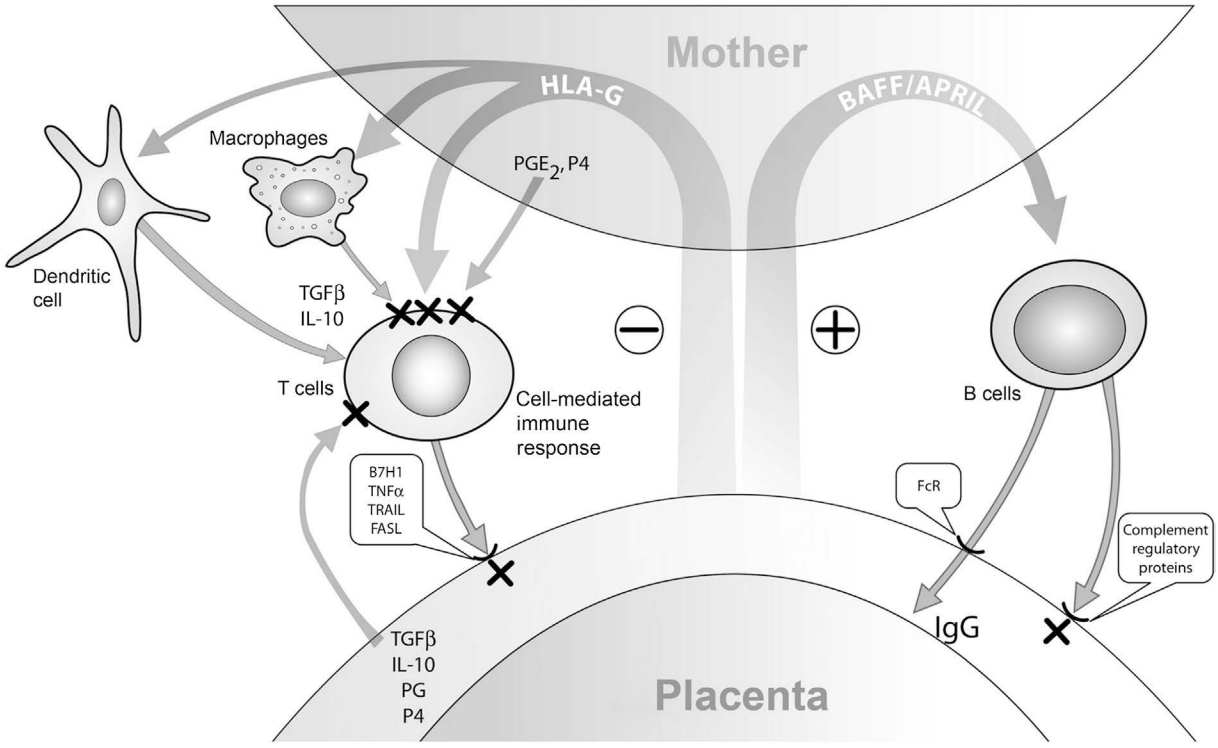


FIGURE 41.5 Placental contribution to tolerance. In this scheme, HLA-G along with immunosuppressive cytokines, prostaglandins, and progesterone, inhibit maternal cell-mediated immunity. Effector cells are impaired by special adaptations on the placental syncytiotrophoblast that include expression of inhibitory cytokines. Antibody-mediated immunity may be promoted by nonapoptosis-inducing members of the TNF superfamily of ligands, BAFF and APRIL. Cytotoxic antibodies directed to placental antigens are inhibited by expression of the complement regulatory proteins on syncytiotrophoblast while beneficial IgG is transported into the fetus.

of maternal uterine blood vessels.[240] In humans, intervals between pregnancies are lengthier when mothers and fathers are genetically similar.[241] The practice of immunizing potential mothers with their husband's or third-party leukocytes is based on the concept of boosting adaptive immunity to paternal MHC antigens, but double-blind studies show this is not a useful technique and may even be dangerous.[242] Because of poor pregnancy outcomes, the practice is prohibited in the United States by the Food and Drug Administration except under approved experimental protocols.

Regulatory T Cells

The most functionally important T cells in the decidual tissue may be immune suppressive T_{reg} cells, defined as $CD4^+CD25^+$ cells expressing the signature transcription factor FOXP3. This special subset of T cells is now believed to play a central role in mediating immune tolerance in pregnancy.[15] Treg cells are powerful inhibitors of inflammatory immune events and type 1 (cell-mediated) immunity.[17] They are critical for preventing immunity to self-antigens as well as exogenous antigens.[243,244] Their suppressive function is essential in tissues containing proteins and other antigenic structures that are not identified as self because of their tissue-restricted expression or occurrence late in development. This is also the case in mucosal and epithelial surfaces, where environmental agents are regularly encountered and tolerance of nondangerous foreign entities is essential to homeostasis and function. This unique capacity to suppress responses to tissue-specific antigens and alloantigens strongly suggests that Treg cells are able to perform a key role in pregnancy tolerance. It is therefore not surprising that $CD4^+CD25^-$ T lymphocytes are now widely believed to be critical suppressor cells that sustain semi-allogeneic pregnancy.[15]

Initially, suppression of activation and proliferation of $CD4^+$ T cells was thought to be the principal function of Treg cells.[245] However, Treg cells are now known to inhibit proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T cells,[246] to suppress B-cell proliferation and immunoglobulin production,[247] to inhibit cytotoxic function of NK cells,[248] and to inhibit maturation and inflammatory activation of antigen-presenting cells including dendritic cells and macrophages.[249–251] Adoptive transfer of wild-type Treg cells but not Treg cells from TGFβ-null mutant donors into nude mice suppresses NKG2D expression and NK cell cytotoxicity, showing the key role for TGFβ in mediating this suppressive function.[248] In dendritic cells, the actions of Treg cells in maintaining tolerogenic dendritic cells and suppressing formation of immunogenic dendritic cells require diverse cell–cell

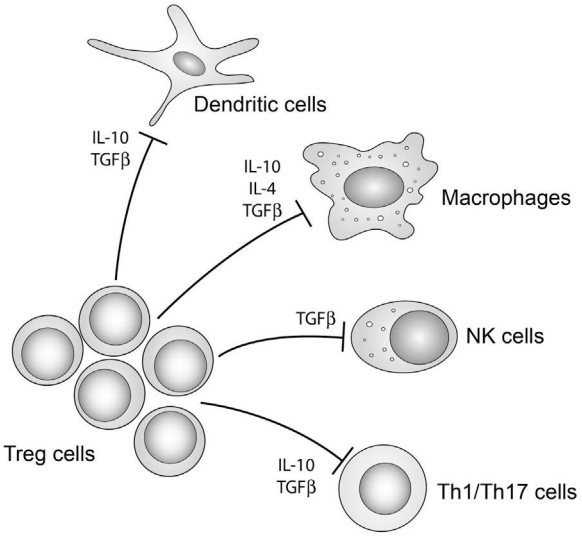


FIGURE 41.6 Suppressive pathways of Treg cells. Treg cells act to exert their immune regulatory and anti-inflammatory effects through inhibiting proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T cells, suppressing cytotoxic function of NK cells, and inhibiting maturation and inflammatory activation of dendritic cells and macrophages. IL, interleukin; TGF, transforming growth factor.

contacts involving CD223 (lymphocyte-activation gene 3, LAG-3), neuropilin-1, and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), which downregulates CD80 and CD86 co-stimulatory molecules. Other soluble signals include IL-10 and TGFβ.[252] Thus, Treg cells can target several stages of the innate and adaptive immune response, spanning the events of lymphocyte activation and proliferation, through to effector function (Figure 41.6). Each of these anti-inflamamtory and immune suppressive functions contributes to their key role in maintaining semi-allogeneic pregnancy.

There are two different pathways of Treg generation. "Natural" Treg cells (nTreg cells) are derived in the thymus, via a selective process based on the TCR structure and its affinity for self-structures expressed in thymic epithelium. Adaptive or "inducible" Treg cells (iTreg cells), which are most important in pregnancy, are produced within peripheral tissues by a mechanism that generates Treg cells with TCRs that recognize antigens restricted to specific peripheral tissues, none of which are present in the thymus.[244] Inducible Treg cells are critical for controlling immunity against foreign antigens that are innocuous, against which aggressive responses would be inappropriate. Under normal circumstances these include reproductive antigens, such as those expressed by the developing conceptus, sperm, or oocytes.

In order to proliferate, become mature, and exert suppressive activity, Treg cells must interact with antigen presented by tolerogenic dendritic cells, in the context of specific immune-deviating environmental signals[62,245] (Figure 41.7). Appropriate cytokines are particularly important for fate commitment of naïve Th0 cells into Treg cells, as opposed to Th1, Th2, or Th17 phenotypes. TGFβ is one critical cytokine, well known for its immune suppressive and anti-inflammatory actions.[253] When TGFβ is present at the time of TCR-antigen ligation, naïve Th0 cells are directed away from the default pathway that generates T cells without suppressive capabilities.[243,244] Instead, these cells differentiate into a suppressor T-cell phenotype and express *Foxp3*.[254] TGFβ can also drive proliferation of mature Treg cells by modulating the function and signaling capabilities of dendritic cells.[255] In vitro experiments show that PGE_2 may synergize with TGFβ to enhance the generation and inhibitory capacity of human $CD4^+CD25^+$ Treg cells.[256]

Once activated and fully functional, Treg cells can interact with other cells in their local vicinity to exert suppression in an antigen-nonspecific manner. Several different and redundant effector mechanisms are responsible for this suppressive activity.[257] Ligation of Treg surface molecules, namely CTLA-4, membrane-bound TGFβ, and CD223, mediate contact-mediated suppression,[257] and this is achieved by competitive sequestration of IL-2 from neighboring T cells.[245,258] Treg cells act to sustain and strengthen a suppressive immune environment by secreting TGFβ and IL-10, which further expands the Treg cell response,[259] and condition dendritic cells to express IDO and maintain a tolerogenic phenotype[221,260] (Figure 41.7).

With their unique capacity to suppress responses to tissue-specific antigens and alloantigens, Treg cells are eminently suited to perform critical functions in mediating tolerance to reproductive antigens in pregnancy. Indeed, inducible Treg cells were discovered almost 10 years ago to have key roles in suppressing maternal immune attack against the conceptus,[15] and since then have been implicated in suppressing autoimmunity to other reproductive and gamete-specific antigens expressed by sperm and oocytes.[261,262] The absolute requirement for Treg cells in semi-allogeneic pregnancy was first demonstrated in experiments where complete T-cell populations or populations depleted of $CD4^+CD25^+$ Treg cells were transferred into pregnant T-cell deficient mice.[15] In the absence of Treg cells, semi-allogeneic fetuses were uniformly rejected, while genetically identical (syngeneic) fetuses generally survived.[15] Passive transfer of CD25-reactive PC61 monoclonal antibody to deplete $CD25^+$ cells in vivo provided further support for an essential role for Treg cells in maternal tolerance of semi-allogeneic fetuses.[263,264] Fetal loss and fetal growth impairment is similarly seen in mice when maternal Treg cells are depleted by diphtheria toxin-mediated killing of CD25-expressing cells.[265] In this latter study of syngeneic pregnancies, only male fetuses were lost, revealing the critical importance of maternal

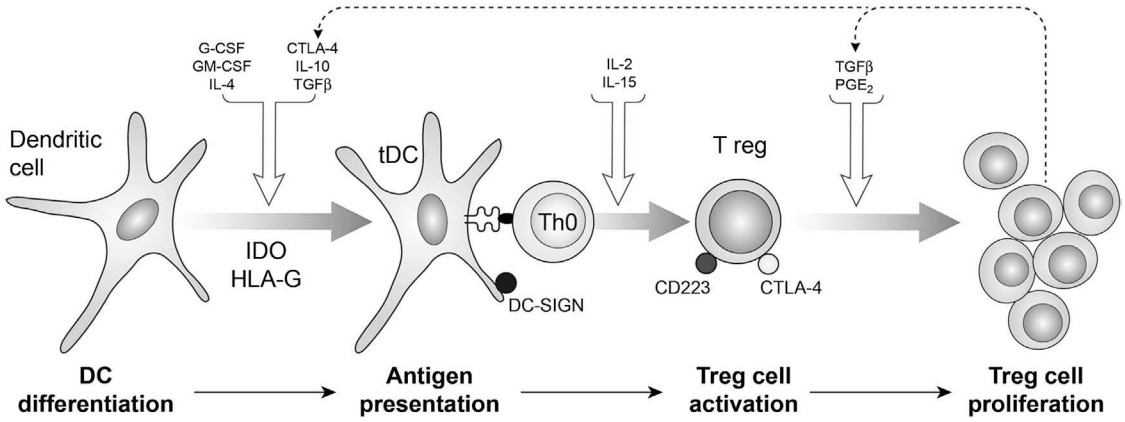


FIGURE 41.7 Suppressive pathways of Treg cells. Tolerogenic dendritic cells, together with regulatory cytokines and other immune-deviating agents, control Treg cell activation and proliferation. Cytokines G-CSF, GM-CSF, IL-4, and IL-10, together with IDO and HLA-G, regulate dendritic cell differentiation into a tolerogenic phenotype. IL-2 and/or IL-15, acting at the site of antigen presentation by tolerogenic dendritic cells, are required for Treg cell activation and proliferation. TGFβ and PGE_2 drive further rounds of proliferation of mature Treg cells. Treg cells release IDO, IL-4, and IL-10 to amplify effects on dendritic cells and Treg cell generation. Ag, antigen; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage CSF; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; TGFβ, transforming growth factor-β; PGE_2, prostaglandin E_2; DC, dendritic cell; tDC, tolerogenic dendritic cell.

Treg-mediated tolerance to antigens encoded by the Y chromosome.

In women, Treg cells comprise approximately 14% of $CD4^+$ cells in early decidua.[126] The decidual Treg cells are phenotypically similar to those in other locations, with expression of intracellular CTLA-4, GITR, and OX40, all of which are markers for this subset. The frequency of $CD4^+CD25^+$ cells in the peripheral blood is increased during early pregnancy,[266] suggesting that cells circulating in blood may migrate into the uterus to increase their numbers during pregnancy. The numbers and suppressive function of these cells in the human decidua are related to the extent of HLA-C mismatch,[11] providing a potential explanation for why MHC disparity between the fetus and the mother can improve pregnancy outcome.[240]

Changes in Treg cell populations are a very early event in the maternal immune adaptation to pregnancy. Rising progesterone levels at the time of embryo implantation are implicated in sustaining and expanding Treg cell populations beyond the estrogen-regulated populations present at ovulation. In vitro, progesterone has been shown to convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Treg cells. This conversion has been demonstrated in vitro using the progesterone antagonist RU486 and in vivo, using progesterone-treated ovariectomized and pseudopregnant mice, suggesting that progesterone expands Treg populations via nuclear progesterone receptors.[27] Progesterone may act to stabilize the Treg cell phenotype and inhibit conversion into pro-inflammatory Th17 cells exposure by inhibiting expression of the IL-6 receptor.[28]

In mice, within two days following mating, an expanded Treg cell pool is detectable in lymph nodes draining the uterus. Similar but less profound changes are seen in the spleen and distal lymph nodes.[15,26,267] In addition to the effects of ovarian hormones, experiments employing tetramer-based strategies to identify T-cell receptor specificity have revealed that pregnancy expands Treg cells with specificity to fetal antigen.[268] As in humans, expression of paternal and other pregnancy-specific antigens in the placenta leads to a greater increase in Treg cell numbers, and is associated with enhanced specific suppression of antipaternal alloantigen reactivity.[267,269] This pre-implantation increase in Treg cells is accompanied by paternal alloantigen-specific functional tolerance, which can be measured as hyporesponsiveness in the Th1 immunity required to elicit a delayed type hypersensitivity response to paternal MHC, or to reject tumor cells expressing paternal MHC.[26]

This early expansion of Treg cell populations in lymph nodes is accompanied by accumulation of $FOXP3^+$ cells and elevated *Foxp3* mRNA expression in the uterus.[15,53] Treg cell recruitment in the uterus during the pre- and peri-implantation period is linked with glandular and luminal epithelial cell production of the Treg cell chemokine CCL19, which acts through the CCR7 receptor to regulate Treg cell recruitment and retention in peripheral tissues.[53] Other chemokines are likely to be involved in attracting Treg cells into the implantation site. Activation by paternal antigens confers expression of chemokine receptor CCR5 on Treg cells, and CCR5 is implicated in sequestering activated, antigen-specific Treg cells in the gravid uterus.[269]

Compelling evidence that Treg cells are most critical at the implantation phase of pregnancy as opposed to later stages comes from Treg cell depletion experiments, which show that semi-allogeneic pregnancy cannot

commence unless sufficient Treg cells are present in the uterus. Passive administration of antibody reactive with CD25 prior to implantation on day 2.5 post-coitum results in implantation failure, while depletion on days 4.5 or 7.5 post-coitum does not terminate pregnancy but instead increases the rate of later fetal resorption. Conversely, depletion in the mid- and late gestation phase of pregnancy does not adversely affect ongoing fetal development.[270]

Confirmation that paternal antigen-specific Treg cells are inducible as opposed to natural Treg cells comes from studies showing their generation depends on the Foxp3 enhancer gene, conserved noncoding sequence-1 (CNS1).[271,272] Increased rates of fetal resorption in CNS1-null mutant females mated with allogeneic males but not syngeneic males confirms that inducible Treg cells modulate maternal responses to paternal alloantigens in gestational tissues.[271] The fact that the CNS1 enhancer emerged in eutherian mammals, where placental tissue is closely interdigitated with maternal tissue, indicates that the CNS1-dependent pathway of extrathymic Treg generation may have arisen under selective pressure to enforce tolerance towards paternal alloantigens and allow viviparous reproduction to evolve.[271]

Chemokines and Selective Decidual Leukocyte Recruitment

Regulated patterns of chemoattractive chemokines synthesized by decidual cells and trophoblast cells in the early developing placenta, together with specific pathways of lymphocyte recruitment, are responsible for the distinct patterns of leukocyte populations present at the fetal–maternal interface.[46,51,273–275] For example, CCL3 (monocyte inflammatory protein-1, MIP1α) has been identified as a major product of cytotrophoblast cells that attracts monocytes and NK cells into the human uterus,[273] whereas IL-8 attracts neutrophils and other leukocyte populations.[276] Interestingly CCL3 is also implicated in facilitating cytotrophoblast cell differentiation,[274] a finding that illustrates the multifunctional nature of immune-regulatory molecules at the fetal maternal interface.

The subsets of lymphocytes recruited into decidual tissue are further restricted by virtue of lymphocyte homing mechanics. All lymphocytes are programmed during their development to follow specific migration pathways through the body that enable antigen-specific immune responses to be concentrated at certain sites. Directed migration, or "homing," into mucosal tissues is controlled by expression of distinct patterns of adhesion molecules on the lymphocyte cell surface, which mediate differential recognition and adherence to endothelium in mucosal sites. The molecular regulation of T-lymphocyte homing into the decidua may be influenced by local microdomains evident within the implantation site, with differential expression of adhesion molecules among local vessels.[277] One example of this is mucosal vascular addressin (MAdCAM-1), which is found in only a proportion of vessels at the leading edge of trophoblast invasion in women, and is otherwise absent from the reproductive tract.[278]

Certain chemokines are involved in recruitment of the Treg cells that are essential for embryo implantation. Accumulation of these cells commences prior to implantation, with experiments in mice implicating estrogen-induced expression of chemokines that target the CCR5 chemokine receptor, including CCL3, CCL4, and CCL5.[24] In the pre-implantation phase, expression of the Treg chemokine CCL19 increases in uterine glandular and epithelial cells in women.[52] In mice, exposure to seminal fluid acts to elevates CCL19 expression.[53] CCL19 acts through the CCR7 receptor to regulate Treg cell retention in peripheral tissues such as the pregant uterus.[279]

The specific patterns of chemokine expression are in part controlled by epigenetic silencing of decidual tissue expression of chemokines that recruit certain T-cell lineages.[132] A mechanism involving repressive histone marks that actively prevent decidual cell synthesis of specific chemokines involved in cytotoxic Th1 cell recruitment, including CCL5 as well as CXCL9, CXCL10, and CXCL11, prevents access of Th1 cells into the decidua in a fail-safe mechanism to ensure that potentially aggressive T cells cannot reach placental tissue. A consequence of this altered chemokine silencing may be to increase the susceptibility of the decidua to infection. Since CXCL10 is also only focally expressed in the human decidua,[280] it is possible that a similar silencing mechanism operates in women.

IMMUNOLOGY OF PREGNANCY: THE FETAL CONTRIBUTION

Sites of Fetal Contact with Maternal Cells

An important mechanism by which the fetus avoids eliciting maternal immune rejection is by tightly controlled expression of immunostimulatory and immunosuppressive molecules. The need for specific immunomodulators is dictated by the anatomical positioning of the trophoblast subpopulation. The direct supply of arterial blood to the placenta throughout the latter two-thirds of pregnancy means that the potential for contact between the syncytiotrophoblast and circulating lymphocytes is constant, and that the syncytiotrophoblast is conceivably the most vulnerable to the maternal immunological milieu. Studies in the mouse have documented both hormone-mediated and fetal antigen-specific curtailment of central T- and B-cell production

during pregnancy, but the mother maintains peripheral immunocompetence and overall levels and functionality of circulating lymphocytes remain intact.[281,282] Further, as reviewed above, the stroma of the decidua basalis is replete with maternal immune cells, constituting about 40% of total cellularity, and extravillous trophoblast cells exiting cell columns stream into the decidua here in vast numbers. Similarly, the chorion membrane resides in direct apposition to decidua cells, and maternal leukocytes can be found at this location throughout pregnancy. Lastly, it has become increasingly clear that yet another maternal–placental–fetal interaction exists within the periphery. As described above, the placenta releases copious quantities of microvesicles directly into the maternal blood, and fetal cell trafficking results in long-term microchimerism in the mother. These vesicles and cells carry both fetal antigens and immunomodulatory proteins directly to maternal lymphoid organs and other tissues. Thus, the varying maternal compartments in which feto-placental cells and vesicles reside necessitate site-specific tailoring to cope with maternal immune cells to which they are exposed.

Regulated Expression of Immunomodulatory Molecules by Trophoblast Cells

HLA Molecules

The major transplantation antigens first identified for their dominant roles in graft rejection are known as the human leukocyte antigens (HLAs). The telomeric end of human chromosome 6p21 (mouse chromosome 17) contains a cluster of these genes (Figure 41.8) (reviewed in Refs 71, 283, and 284). Within this cluster are 16 HLA class I genes, only six of which are transcribed or translated.[285] The class I genes that are expressed by cells are subdivided into the highly polymorphic class Ia, which includes HLA-A, -B, and -C genes, and the weakly polymorphic class Ib genes, which include HLA-E, -F, and -G genes. HLA class II (HLA-D) genes are also highly polymorphic. Regulation of the genes encoded by the three groups differs greatly, being highly cell- and tissue-specific as well as related to the differentiation and activation states of cells. The antigens are functionally versatile, driving either immunostimulatory or immunoinhibitory pathways. In particular, when released in the soluble form, these antigens are perceived as immunosuppressive rather than immunostimulatory (reviewed by Zavazava[286]).

The HLA class I genes are expressed biallelically, such that the protein products of both maternally derived and paternally derived HLA are present on individual cells. Class Ia antigens exist as membrane-bound molecules on the surface of most nucleated cells, but can also exist in the serum in a variety of soluble isoforms (reviewed by Campoli and Terrone[287]). Their expression is not cell type-restricted; HLA-A, -B, and probably also -C are expressed by essentially all somatic cells. These antigens are unique markers of individual identity as each gene is highly polymorphic, with thousands of alleles each.[285]

By contrast, products of the class Ib genes are held in common among most individuals. The numbers of alleles are low for HLA-E and HLA-F, and the number of proteins with differing amino acid compositions are 5 and 4, respectively. To date, 50 alleles of HLA-G encoding 16 distinct proteins have been identified.[285]

TROPHOBLAST REPRESSION OF HLA EXPRESSION

Several decades of investigation have shown that syncytiotrophoblast cells may either fail entirely to express HLA antigens or may select specific genes with special nonimmunogenic properties for expression (reviewed in Refs 71, 283, and 284). The antigens that trophoblast cells never express in vivo are HLA-A and -B, which in other contexts are highly effective stimulators of acute graft rejection. Trophoblast cells also effectively repress expression of HLA class II antigens,[288] which are used for stimulation of the T-helper cell subset. Although some types of cells can be induced to express HLA class Ia and class II antigens, trophoblast cells in placental explants cultured in vitro cannot.[289] When released from the architectural environment of the normal placenta, purified trophblast cells can be induced to express

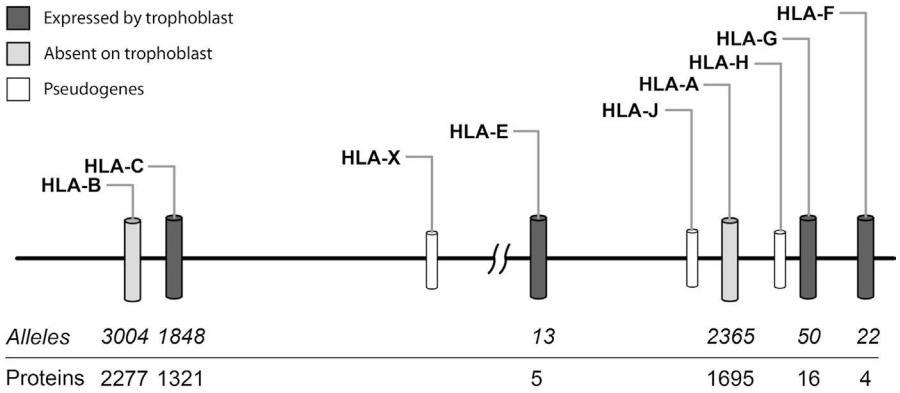


FIGURE 41.8 The human major histocompatibility on chromosome 6. The HLA class I genes, which are telomeric to HLA class II, include HLA class Ia (HLA-A, -B, -C) and Ib (HLA-E, -F, -G), and trophoblast cells strictly regulate the expression of each of these genes. Three of the 10 known, unexpressed HLA pseudogenes are shown. Each of the class I genes differ significantly in the numbers of alleles known and proteins expressed (bottom panel). HLA, human leukocyte antigen.

class I HLA with inflammatory cytokines,[290] suggesting that elements within the placenta have a major role in regulating expression of HLA in trophoblast cells. This robust repression of HLA-A, -B, and class II molecules prevents trophoblast cells from participating in antigen presentation and from comprising direct targets of $CD8^+$ and $CD4^+$ T cells. Thus, this mechanism has long been recognized to be a major pathway whereby the placenta passively escapes maternal immune rejection.

TROPHOBLAST HLA-C

Human extravillous trophoblast cells express one class Ia molecule, HLA-C, and all three class Ib molecules. The HLA-C gene is polymorphic, and could stimulate maternal antifetal acquired immunity if paternal alleles differed from maternal. As discussed above, trophoblast HLA-C may be recognized by maternal cells, and provoke the need for an immunosuppressive Treg response.[11] Thus allelic disparity at the HLA-C locus is not usually a causal factor in infertility or termination of pregnancy. While increased infiltration of paternal HLA-C-specific T cells into the maternal–fetal interface is associated with higher allelic disparity, these cells may be kept in check by corresponding increases in local Treg cells.[11]

Accumulating evidence suggests that HLA-C has a trophic function in placentation, specifically by promoting uNK cell participation in remodeling the uterine spiral arteries—the critical supply line delivering maternal blood to the developing placenta. Experimental evidence supporting this idea has been growing for more than 15 years, starting with the seminal findings of failed spiral artery remodeling in mice lacking NK cells.[291] The narrow, smooth muscle-bound decidual arteries in these mice is reminiscent of the restricted spiral artery remodeling found in pregnancy disorders such as preeclampsia and intrauterine growth retardation.[292,293] Successive studies using mouse models, purified cells ex vivo, and in vitro models have demonstrated that uNK cells display a unique synthesis profile of pro-angiogenic growth factors, trophoblast migration-promoting chemokines, and smooth muscle cell disrupting factors.[196,294–297] Further, extravillous trophoblast and uNK cells appear to engage in bidirectional cross-talk. A principal trophoblast-derived factor in engaging uNK cells appears to be HLA-C, recognition of which uNK cells are biased towards.[298] Epidemiologic evidence supports this model: Hiby and co-workers have reported that combinations of genotypes of maternal killer inhibitory receptor (KIR) and fetal HLA-C that should, at the molecular level, encourage NK activation, act to reduce risk of preeclampsia and pregnancy failure.[299–301] Thus, a growing body of literature supports the concept that activation of uNK cells through trophoblast HLA-C stimulates production of cytokine and growth factors, which in turn promotes trophoblast migration and disarrangement of smooth muscle cells encircling spiral arteries. Although the cellular and molecular mechanisms of these genetic associations are as yet undefined, further studies seeking to reveal the role of placental HLA in maternal spiral artery remodeling are warranted, including those using murine models.[240]

CLASS IB HLA: HLA-E, HLA-F, AND HLA-G

The other HLA class I antigens expressed by trophoblast cells are HLA-E, -F, and -G. The importance of HLA-E is discussed above in the section on uNK cells. Although there was originally much confusion regarding the specific class I HLA that signaled inhibition of uNK cells, it is now clear that HLA-E, which binds to the CD94/NKG2 receptor, is of prime importance in preventing uNK cells from killing their normal targets.[193] However, HLA-G might yet prove of value in this setting because the uNK cells express both leukocyte immunoglobulin-like receptor B1 (ILT2 or LILRB1) receptors and KIR2DL4, which signal inhibition through different pathways. Figure 41.9 shows the potential immune cell targets of trophoblast cell HLA-G antigens and the receptors known to bind the specific antigens. HLA-F is also present on invading cytotrophoblast cells,[302,303] but its binding patterns and activities remain unknown.

The physical properties of HLA-G are unique; two of these profoundly influence its regulation and functions. First, the gene has a large deletion in its promoter region that precludes enhancement of expression by the usual modulators, i.e., interferons and TNF. Second,

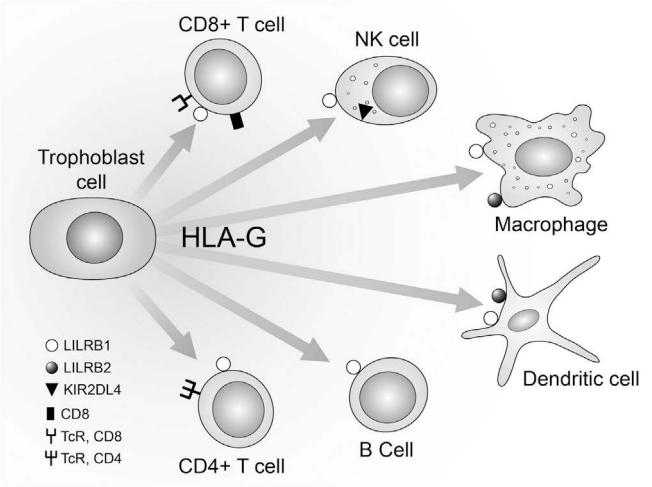


FIGURE 41.9 Trophoblast HLA and immune cell interactions. Each subpopulation of leukocytes expresses its own pattern of receptors for HLA class I, which permits diversity in responses. Note that the leukocyte immunoglobulin-like receptors (LILR) comprise a large family, and that individual leukocyte subgroups express specific members such as TCR (T cell receptor), ILT (immunoglobulin-like transcript), or KIR (killer inhibitory receptor).

the HLA-G transcript is alternatively spliced to yield seven different messages, of which four are predicted to encode membrane-bound proteins and three are predicted to encode soluble proteins (reviewed in Refs 71, 283, and 284).

The placental expression patterns for HLA-G have been extensively investigated and reviewed.[71,304] HLA-G was first identified on the term chorion membrane and on cytotrophoblast cell columns in early pregnancy. The invasive interstitial and endovascular cytotrophoblast cells remain HLA-G$^+$. Further, individual isoforms derived from the HLA-G gene by alternative splicing have varying expression patterns. The membrane-bound isoform that is derived from the full-length message, HLA-G1, appears on invasive cells, but smaller isoforms that may be either membrane bound or soluble (HLA-G2 and -G6) are also prominent on the extravillous cytotrophoblast cells.[69] By contrast, a second soluble isoform, HLA-G5, is ubiquitous in placentas and membranes taken from all three trimesters of pregnancy.[305]

The membrane-bound form that has been most thoroughly studied is the full-length protein, HLA-G1. This isoform is present on invading cytotrophoblast cells in columns distal to the placental villi, on interstitial and endovascular trophoblast cells, and on some cells remaining in the term chorion membrane. HLA-G1 and its soluble counterpart, HLA-G5, can exist as free heavy chains or as heterodimers, each in association with β2-microglobulin. This latter form further homodimerizes via disulfide linkage between the heavy chains; dimerization confers increased affinity and bioactivity for its LILRB1 receptor.[306–309] Its target could be uNK cells through LILRB1 and/or KIR2DL4 receptors; LILRB1 and LILRB2 molecules likely mediate HLA-G interaction with T cells and macrophages, respectively (Figure 41.9). HLA-G can inhibit antigen-spectific cytolysis by cytotoxic T cells and NK cells,[310] as well as CD4 T-cell alloproliferative responses and cytokine production.[311,312] HLA-G also could modulate T-cell activity indirectly through its LILRB2-mediated actions on dendritic cells.[313] Interestingly, HLA-G1-transfected antigen-presenting cell lines have been shown to display enhanced levels of receptors such as KIR2DL4, LILRB1, ILT3, and LILRB2,[314] which could comprise a feed-forward pathway for secretion of cytokines or alteration of other antigen presenting cell activities.

The functions of HLA-G5 might be similar to those of HLA-G1, as their structures are identical except that HLA-G5 is missing the transmembrane and cytoplasmic domains and is therefore a soluble protein. Binding of HLA-G5 to receptors on leukocytes has profound effects, and these are exerted through multiple pathways. HLA-G5 stimulates death of phytohemagglutinin (PHA)-treated lymphocytes through the Fas/FasL cell death pathway, reduces expression of lymphocyte CD8α, a co-receptor involved in linking CTL with antigen-presenting cells, and drives mononuclear phagocytes into an immunosuppressive profile where the cells produce high levels of TGFβ (reviewed in Refs 7, 11, and 67). Soluble HLA-G appears to be selective in its enhancement of suppressive cytokines; there is no relation, for example, between levels of HLA-G and IL-10 in serum.[315] The first report of binding of HLA-G to macrophages indicated a preference for LILRB2, but studies in U937 cells, a histiocytic cell line, indicate that both the LILRB1 and the LILRB2 receptors on these phagocytes bind HLA-G5.[175]

Evidence is accumulating for functional distinctions among the isoforms. The argument for similar functions is supported by the finding that in the absence of HLA-G1 and -G5 due to a deletion in the HLA-G gene, women have demonstrable HLA-G proteins in their placentas and produce viable offspring.[316–318] These results are consistent with the idea that other isoforms provide functional compensation. Although initially controversial, several studies show that these are not only present in placentas but are functional.[305,310,319,320]

The argument for isoform-specific functions is supported by the report that HLA-G2/G6 is expressed exclusively in the extravillous cytotrophoblast cells distal to the villus whereas HLA-G5 is ubiquitously expressed in villous and extravillous cytotrophoblast cells and is prominent in syncytiotrophoblast and maternal blood at term.[305,321] At this point in time, the major isoform-specific distinction supported by experimental evidence is concentration-dependency; HLA-G5 is a significantly more effective stimulator of certain responses such as production of TGFβ by macrophages[175] than is HLA-G6 (reviewed in Ref. 71). However, macrophage-like cells demonstrate a significant preference for binding the HLA-G6 isoform via their LILRB1 receptors whereas binding of the HLA-G5 isoform is performed equally by LILRB1 and LILRB2,[175] suggesting that discrete, receptor-driven, isoform-specific functions may yet be uncovered. The functions of HLA-G2, HLA-G3, and HLA-G4 remain poorly explored with the exception of a report from Riteau and co-workers showing that as with HLA-G1, all of these isoforms protect transfected cells from effector cell lysis.[310,320] The expression and fuction of HLA-G7 remains unknown.

B7 Family Co-stimulatory Molecules

T cells require two signals from antigen-presenting cells to become fully activated. As well as the antigen-specific signal provided through T-cell receptor ligation with HLA-peptide complex, a second signal, the co-stimulatory signal, is required. Co-stimulation is antigen nonspecific and is provided by the interaction between co-stimulatory molecules expressed on the membrane of the antigen presenting cell and the T cell. One of the best characterized co-stimulatory

molecules expressed by T cells is CD28, which interacts with B7-1 (CD80) and B7-2 (CD86) on the membrane of antigen-presenting cells (reviewed by Greenwald et al.[322]). Without the co-stimulatory signal, naive lymphocytes, instead of becoming activated in response to the antigen/HLA complex, may become tolerized and anergic to subsequent stimulation.[323] With both signals, however, naive lymphocytes are primed to respond to antigenic stimulus, and the result is proliferation and cytokine production. Conversely, B7-1 and B7-2 may down-modulate T cell activation through the CTLA-4 receptor, which serves to shut off the immune response. The limitation of expression of B7-1 and B7-2 to professional antigen-presenting cells ensures that only cells presenting foreign antigen, and not cells expressing only self-antigen, can elicit an immune response.

There are currently seven known members of the B7 family, at least five of which are transcribed and translated in the human placenta.[208] There are also five known CD28 family molecules. Depending in part on the receptor–ligand combination, these interactions have either inhibitory or stimulatory action on immune cells.[324,325] In contrast to the restricted expression of B7-1 and B7-2 on antigen presenting cells, other members of the B7 family have a broader range of distribution, at least at the level of mRNA, and many cells can express these proteins upon induction by inflammatory stimuli.[326,327] Uniquely, all human trophoblast cells abundantly and constitutively express the B7 protein, B7-H1 (or PDL1); its cognate inhibitory receptor, PD-1, is abundant on decidual $CD8^+$ and $CD4^+$ T cells, including $CD4^+CD25^+FoxP3^+$ Treg cells.[328]

The cellular distribution of the more recently described members of the B7 family on both antigen-presenting cells within lymphoid organs and on parenchymal cells in the periphery reflects their ability to modulate T-cell activation at both the priming and the effector stages. Due to the absence of class Ia HLA on trophoblast cells, this protein most likely modulates effector activity of T cells in the placenta. In vitro, trophoblast B7-H1 shifts cytokine production by activated human decidual T cells towards a Th2 profile.[208,328] Whether the B7-H1/PD-1 pathway is requisite for semi-allogeneic murine pregnancy is controversial; while a requirement for maternal B7-H1 has been reported, results are inconsistent among investigators; furthermore the B7-H1 receptor PD-1 is not mandatory.[329,330]

2,3-Indoleamine Dioxygenase

In 1998, Munn et al. presented evidence in a mouse model suggesting that inhibition of tryptophan metabolism by placental cells via production of IDO comprises a major strategy for protecting the embryo from maternal immune cell attack.[331] The experiments described a system in which chemical inhibition of IDO, an enzyme which removes tryptophan from the environment and is a known inhibitor of cell proliferation, caused fetal rejection at gestation day 10 that was entirely restricted to semi-allogeneic pregnancies. More recently, the group of investigators who generated the first report on placental IDO demonstrated that in the mouse placenta, IDO is located in trophoblast giant cells.[332]

Evaluating the role of IDO in human pregnancy is difficult because women deficient in this ubiquitous enzyme are unknown. Studies on human placentas and decidua have yielded conflicting results, with placental endothelial cells, isolated stretches of syncytiotrophoblast, placental mesenchymal cells, extravillous cytotrophoblast cells, and placental macrophages identified as positive by immunohistochemistry.[333,334] Whether human decidual macrophages are IDO positive or negative remains in doubt, but stage of gestation may be important.[172,335] Many other types of leukocytes, including eosinophils,[336] dendritic cells,[337] and Langerhans cells,[338] also express IDO, but none has been investigated in pregnancy. The assortment of results and their probable contributions to human semi-allogeneic pregnancy have been discussed by Entrican.[339]

Evaluating IDO functions in mice is also difficult. In contrast to their predictions, Baban and co-workers found that mice with genetic deficiencies in IDO have entirely normal pregnancies.[332] The investigators postulated that other mechanisms of immune suppression compensated for lack of IDO, but the experiments could also be interpreted to mean that IDO is not an immune modulator in pregnancy. Bonney and Matzinger[134] expanded the Danger Theory to this concept, raising the question of whether or not, in the original experiments,[331] the IDO-reducing chemical simply damaged fetal cells which then emitted the danger signals associated with dead and dying cells. These signals stimulated an influx of paternal antigen-specific and antigen-nonspecific leukocytes that ultimately destroyed the placenta and fetus. In summary, while intriguing, the importance of this molecule to the immunology of pregnancy remains to be clearly established.

TNF and TNF Receptor Superfamilies

The Fas/FasL pathway was first implicated in maternal–fetal tolerance more than 15 years ago when the death-inducing ligand was found in human trophoblast cells and its receptor on decidual leukocytes.[340] It was soon posited as a mechanism to deter access of leukocytes into the decidua: matings of FasL-deficient *gld* mice, in which both fetus and mother lack functional FasL, gave small litter sizes and substantial fetal resorption accompanied by infiltration of leukocytes into the placenta.[341] Although later studies revealed a likely strain-dependency of this effect, data in human in vitro models substantiate the initial hypothesis. FasL-expressing human

primary trophoblast cells were found to kill nonspecifically activated leukocytes through ligation of cell surface Fas receptor.[342] FasL is also expressed within the decidua, and maybe co-opted for use in removal of endothelial and/or vascular smooth muscle cells during spiral artery remodeling.[343]

Initially assumed to be expressed at the cell surface, FasL is now known to be contained within vesicles in the cells and released via association with exosomes.[145,343] This is true for both interstitial trophoblast cells and syncytiotrophoblast, consistent with the idea that it can act both locally at the maternal–fetal interface, and systemically following secretion from the chorionic villi. Indeed, FasL is found in association with exosomes in maternal peripheral blood, and in mice, is required for deletion of fetal antigen-specific T lymphocytes.[345] Mechanistically, Fas/FasL may be an intermediary for several immune privilege-promoting molecules including HLA-G and hCG, as it plays at least a partial role in death induced by these molecules.[346-348] Fas receptor is on decidual CD4+ and CD8+ leukocytes, and inverse positioning of FasL-positive decidual cells and Fas receptor on leukocytes. Fetal FasL is necessary for deletion of fetal antigen-specific T cells in the mouse.[349]

There are many other members of the TNF superfamily and TNF receptor superfamily: 19 and 29 to date, respectively, and each may play a specific, equally significant role in maternal–fetal immunology. Many are transcribed and translated in the human placenta,[350,351] and can be detected as soluble factors in human pregnancy serum. Like FasL, TRAIL (TNF-related apoptosis-inducing ligand) is highly expressed by trophoblast cells, is released into maternal serum, and is toxic to leukocytes bearing its receptors.[352,353] Similarly, APRIL (a proliferation-inducing ligand), BLyS (B lymphocyte stimulator), and CD30L are present.[232] Provocatively, trophoblast cells often express receptors for TNF superfamily proteins, but have intact mechanisms for resistance to apoptosis induced by these ligands. Thus, trophic functions of these proteins in promoting trophoblast survival or function are probable and indeed confirmed for the flagbearer member of this family, TNF, in both human and mouse models (reviewed by Haider and Knofler[354]).

TNF family proteins may also play a role in modulation of antibody-mediated immunity during pregnancy. While researchers have focused mainly on the problem of how cell-mediated responses are diminished, the question of how antibody levels become elevated in pregnant women has not been thoroughly investigated. Yet this question is of great importance, as newborns need a broad spectrum of antibodies for protection from newly encountered environmental pathogens. While mothers continually generate antibodies that circulate in the blood, studies using transgenic mice have shown that production of B cells capable of producing antifetal antigen antibodies is dramatically deterred.[282] Ultimately, noncytotoxic IgG antibodies are delivered to the fetal circulation via special neonatal Fc receptors on syncytiotrophoblast,[355,356] an event paramount to passively immunizing the fetus and neonate against microbial infection.

The placenta is actively involved in promoting synthesis of immunoglobulin. Human trophoblasts produce two nonapoptosis-inducing TNF superfamily ligands, BAFF and APRIL, with the capacity to sustain B-lymphocyte viability (Figure 41.5).[232] An unfortunate consequence is that mothers with antibody-mediated autoimmune disorders may suffer more during pregnancy. These ligands are elevated in women with autoimmune diseases, and have been suggested to comprise a major underlying defect in their homeostatic mechanisms.

The superabundance of maternal antibodies might be expected to have detrimental effects, as some could be cytotoxic to the placenta and extraplacental membranes. Yet placental destruction via antibody-mediated immunity does not take place in normal pregnancies. Mammalian placentas have installed an effective protective mechanism to prevent their destruction by complement-activating cytotoxic antibodies. Human placentas display an array of complement regulatory proteins that interfere with complement deposition and consequent toxicity.[355] Mice display only one of the complement regulatory proteins, and a knockout mouse model has shown definitively that in the absence of this protein, the placenta is destroyed.[357]

IMMUNOLOGICAL ORIGINS OF PREGNANCY DISORDERS

The evidence for associations between immune disturbances and various clinical complications of pregnancy is now overwhelming. Unexplained infertility, recurrent spontaneous miscarriage, preeclampsia, and preterm delivery are all linked with altered immune parameters. One caveat with assigning an immunological origin has been to recognize the distinction between causal events and those that are a consequence of fetal loss; this is particularly difficult to dissect in human clinical conditions. Notwithstanding, the links between altered immune parameters in at-risk women and many of the perturbations shown in mice to cause infertility and pregnancy loss are compelling. In broad terms, a consistent emerging theme is that a shift away from the anti-inflammatory, pro-tolerance environment of normal pregnancy is a risk to ongoing pregnancy success. This can occur due to a range of challenges mediated by exogenous or endogenous pro-inflammatory agents, or perturbed or insufficient anti-inflammatory protective mechanisms.

It is notable that the demise of protective mechanisms linked with attenuated progesterone signaling is the natural precedent of parturition and delivery at term. This raises the prospect that the immune response of pregnancy may be linked with the timing and success of the birth process (see Chapter 42). In support of this, the percentage of $CD3^+$ decidual lymphocytes that express markers of Treg cells are significantly decreased in deciduas in spontaneous vaginal delivery compared to elective caesarean section.[267,358] This implies a potential role for Treg cells in the pro-inflammatory changes during late stages of pregnancy, and prompts speculation that their decline near parturition might be a causal factor in fetal expulsion from the maternal tissues when gestation is complete. However, the premature conversion of Treg anti-inflammatory to pro-inflammatory activity at less than 36 weeks of gestation can have important, lifelong consequences. This has prompted substantial recent efforts to understand how the immune system contributes to preterm labor and related pregnancy complications.

Placental Responses to Infection

As the fetoplacental unit grows, the chorionic villi become increasingly complex and numerous, expanding to accommodate the needs of the growing fetus. The syncytiotrophoblast constitutes the single largest physical interface between mother and fetus—estimated to be up to $12\,m^2$ at term[359]—and this large surface area is essential for many critical functions that include bidirectional transport of nutrients and waste between the mother and the fetus, production of pregnancy-specific hormones, and protection against environmental toxicants.[360] Given this enormous area and particularly the anti-inflammatory and immune suppressive environment conferred by the Treg cells and other agents of maternal tolerance, the gestational tissues in the decidua should be vulnerable to microbial colonization. Indeed it seems remarkable that infection in pregnancy is not more common.[361] Fortunately, the syncytiotrophoblast and villous cytotrophoblast cells serve as a structural and functional barrier to placental and fetal infection by systemically distributed pathogens in maternal blood. The trophoblast layers serve as a first-line of defense against fetal via expression of receptors that recognize bacterial and viral products, including TLR2, TLR3, TLR4, and TLR7/8. The proposition that trophoblast cells contribute critically to resistance to infection by acting as a component of the innate immune system was first put forth by Pollard.[362] These investigators demonstrated in mice that these cells produce chemoattractants to promote a uteroplacental immune response in response to systemic *L. monocytogenes* infection.

The human placenta also may contribute systemic and paracrine mechanisms in its resistance against microbes. Exosomes emanating from the placenta carry the placenta-specific C19MC microRNAs, mirroring the profile of both trophoblast and pregnancy serum-derived microRNAs.[363] These microRNAs have recently been discovered to confer resistance of target cells to infection by a variety of clinically important viruses.[364] This newly described function of placental exosomes may prove to be an important antimicrobial defense mechanism for both mother and fetus.

Deficiency in Treg Cells

Several studies link compromised function or diminished Treg cell populations with complications of pregnancy that originate in defective implantation or placental dysfunction.[16] In broad terms, insufficient Treg cells are likely to dispose towards increased inflammation and, potentially, development of Th1 and Th17 cell subsets (Figure 41.10). In preeclampsia, $CD4^+CD25^{high}$ T cells are significantly reduced in the peripheral blood and decidual tissue compared to women with healthy pregnancies.[365] Changes in decidual Treg cells together

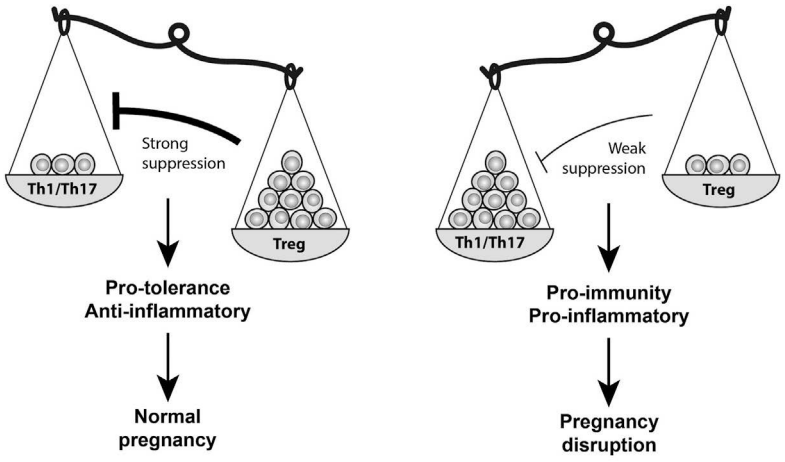


FIGURE 41.10 Treg and reproductive dysfunctions. Deficiencies in Treg cell numbers and/or suppressive function are associated with reproductive disturbances including infertility, recurrent spontaneous abortion, and preeclampsia in women. Current understanding is that adequate Treg cell numbers and function acts to suppress inflammation as well as Th1/Th17-mediated cytotoxic attack on the semi-allogeneic conceptus, but Treg cell depletion leads to insufficient suppression and increased inflammation, with Th1/Th17-mediated fetal loss.

with an elevation in the proportion of pro-inflammatory Th17 $CD4^+$ T cells are reported to be most notable in early-onset severe preeclampsia.[366] These observations are consistent with the idea that an imbalance in the reciprocal functions of regulatory and effector T cells contributes to this disease.[367] Notably, inducible Treg cells, as opposed to natural Treg cells, are diminished in preeclampsia; this may originate in reduced capacity for CD14+DC-SIGN+ antigen-presenting cells to drive Treg cell differentiation.[223]

In miscarriage, reduced immunosuppressive capability may be due to insufficient expansion of Treg cell populations, leading to numerically fewer Treg cells as well as Treg functional deficiency. Both decidual and peripheral blood $CD4^+CD25^{high}$ T cells are diminished when measured after spontaneous abortion compared to induced abortions.[368] Women experiencing repeated miscarriage have a reduced frequency of Treg cells among the peripheral blood $CD4^+$ pool as well as lower suppressive capacity when compared with fertile women.[25] There is also evidence of elevated susceptibility to IL-6 trans-signaling, which can mediate abnormal suppressive function and disposition to Th17 conversion.[369] Low levels of circulating Treg cells in early pregnancy are predictive of miscarriage risk.[370] Conversely, levels of IL-17-producing T cells in the peripheral blood and decidua are increased with signs of imminent miscarriage.[371,372]

Primary unexplained infertility is also associated with reduced endometrial expression of *FOXP3* mRNA,[373] suggesting that impaired differentiation and/or sequestration of Treg cells into the decidua even prior to conception may affect achievement of pregnancy. There is evidence that some chronic disease states that impact systemic immune function such as in asthma,[374] allergy[375] autoimmune disease,[376] and HIV infection,[377] can alter immune adaptation to pregnancy and may specifically reduce Treg cells.

Dysfunction in uNK Cells

Various studies have suggested that there are changes in the numbers, phenotype, and/or function of uNK cells in women who repeatedly miscarry or experience unexplained infertility. Analysis of leukocytes from the nonpregnant endometrium of women who have experienced three or more miscarriages showed an increase in the number of CD56dim CD16+ cells and a decrease in the normal CD56bright CD16- uNK cells.[379] It has been suggested that this shift in the balance of NK phenotypes represents a hostile environment for implantation that is inconsistent with healthy pregnancy progression. Similar findings were reported for midsecretory endometrium in infertile women prior to unsuccessful IVF cycles, compared with successful IVF cycles.[379] Altered activation phenotypes in uNK cells are also evident in decidual tissues recovered at the time of miscarriage.[23,169,380] However, whether this is a causal feature or simply a consequence of the inflammatory events leading to miscarriage, is difficult to determine.

Disturbances to HLA-G

Relationships and potential associations between HLA-G and poor reproductive capacity have been extensively investigated, with mixed results that may be dependent upon diagnostic criteria and/or technical differences in the studies. Data from women with unexplained recurrent miscarriage[381–383] relates the condition to variation in the HLA-G promoter region.[382] Yet Patel and coworkers report that HLA-G on cell membranes is not associated with idiopathic recurrent pregnancy loss.[384]

Preeclampsia has been intensively studied; this disease is marked by abnormal invasion of cytotrophoblast cells into the decidua,[385] suggesting some role for HLA-G, which appears prominent in invasive cytotrophoblast cells. Levels of HLA-G may be reduced in the invasive cells of preeclamptic women.[386,387] Recent studies focusing on HLA-G alleles[388] and isoforms,[389] or combinations thereof,[317] and associations with preeclampsia[390] are present in the scientific literature. Le Bouteiller has reviewed some of the recent observations on HLA-G and preeclampsia.[391] Little attention has been paid to intrauterine growth retardation, perhaps because there is evidence that this condition is not associated with loss of the ability of a mother to synthesize HLA-G1 and HLA-G5.[389]

In summary, specific DNA sequences, whether in the promoter or coding region, appear to control the amounts of HLA-G[392] that are produced. Reduced levels may be associated with disease, yet when the changes result mainly in reduction of specific isoforms, other isoforms may substitute and compensate, permitting pregnancy to proceed.

Although HLA-G was originally believed to be expressed only by human trophoblast cells, this is clearly not the case. HLA-G is also expressed in human macrophages[174] and expression is linked to activation of the macrophages by IFNγ. This was subsequently verified in another laboratory where HLA-G5 was identified in activated monocytes using cell ELISA spot assays.[393] There are many additional reports suggesting a role for inflammation in driving expression of HLA-G. It remains to be determined what the functions of the HLA-G molecules may be in nontrophoblastic cells, but evidence in transplant patients suggests that this molecule, which was first identified as a natural immunosuppressant in pregnancy, may exert protective effects in patients undergoing heart transplants.[394]

CONCLUSION

The last half-century of research effort to understand the immunological enigma posed by semi-allogeneic pregnancy has brought extraordinary advances and revelations. Multilayered and sophisticated strategies for allowing semi-allogeneic pregnancy have been identified and triaged into those that appear essential and others that support and strengthen a healthy coexistence. Importantly, this knowledge is providing a new prism through which to understand the origins of many disorders of pregnancy that can have devastating consequences for children and their families, as well as high societal and health care costs. It seems reasonable to expect that future lifestyle strategies and drug therapies to suppress inflammation, through enhancing tolerogenic antigen-presenting cell functions and/or boosting Treg cell numbers, will ultimately emanate from the emerging understanding of the immune response to pregnancy.

There are several important research questions still to be resolved. Key knowledge gaps are the principal pathways and determinants by which the balance of tolerogenic versus immunogenic leukocytes are activated in a healthy pregnancy, and the most common pathways by which this is disturbed in women with reproductive disorders. A picture in which several environmental and genetic determinants may converge to induce inflammatory stress and shift this balance, is emerging. It seems likely that future research will focus on how dietary factors and micronutrients, obesity, the maternal intestinal and vaginal microbiome, stress, infection, and environmental chemicals may all compromise the immune response to pregnancy through elevating inflammation. Other challenges are to define the significance of HLA antigen dissimilarity or sharing in aberrant leukocyte activation, and the role of progesterone among other molecular mediators in T-cell and NK-cell differentiation. The importance of the immune response as a sophisticated quality control system and how this relates to infertility and miscarriage incidence needs to be resolved. Critically, a solution for preeclampsia can only arise once we fully understand how NK cells, Treg cells, dendritic cells, and macrophages all interact to control placental vascular development in the early phase of placental development. Charting the sequence of events and critical factors that cause leukocytes to become activated, be recruited into gestational tissues, and initiate parturition at the end of gestation is a major challenge that promises important insight into preterm labor and options for its prevention.

Until these issues are resolved and a better understanding of the fundamental biology underlying the maternal immune response in pregnancy is available, it will be essential to remain cautious with unproven experimental interventions such as administration of anti-inflammatory and immune suppressive agents. This is particularly the case for infertility treatments where the risk-benefit analysis of experimental treatments in otherwise healthy, reproductive-aged women is ethically challenging compared to patients suffering terminal illness or a debilitating autoimmune condition.

Furthermore, immunologists have found that the natural situation of pregnancy comprises an excellent model for defining how tumors usurp immune rejection and for devising new strategies that might improve the success rate for transplantation of organs and cells. Traditional therapies have focused on the host, with administration of cytotoxic drugs to the tumor-bearing patient, and modulation of host immune responses in graft recipients. Yet pregnancy immunology has taught experimentalists and clinicians that both the host and the invading tissue cooperate to develop a protected environment. Now, therapies are being devised wherein host immune cells attacking tumors are redesigned to meet the challenge of tumor evasive strategies, and gene-modulated organ and tissue grafts are being constructed to mimic pregnancy-like host tolerance.

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CHAPTER

42

Parturition

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INTRODUCTION

Parturition—the process of birth—is fundamental to viviparity and a pivotal event in the mammalian reproductive cycle. The goal of this chapter is to synthesize current understanding of the physiology and pathophysiology of human parturition from the perspective of (1) the uterus and its functional tissue types: the myometrium, cervix, decidua, and fetal membranes; (2) the hormonal interactions that control the myometrium, cervix, decidua, and fetal membranes; (3) the physiologic interactions that trigger parturition and determine birth timing; and (4) the evolutionary biology of human parturition and the specific and unique selective pressures in the hominid lineage that influenced the extant physiology and pathophysiology of human birth.

The process of parturition is similar in all viviparous species and culminates in the forceful emptying of the uterus to safely deliver the conceptus. Historically, the central role of the hormone progesterone, as necessary to maintain pregnancy and block the onset of labor, has been recognized for more than 70 years. This "progesterone block" hypothesis, originally proposed by George Corner in the 1940s[1] and later refined by Arpad Csapo in the 1950s,[2] remains a cornerstone of parturition physiology. Subsequent in vivo studies in sheep by Liggins and colleagues in the 1960s established activation of the fetal hypothalamic–pituitary–adrenal axis as the driver of withdrawing progesterone (removing the block) and initiating parturition.[3–5] Findings in the sheep, however, proved problematic to extrapolate to human parturition, as systemic progesterone withdrawal, a fall in circulating serum progesterone, does not occur in women before the onset of labor. Similarly, advances in the use of genetically altered mice in the period from the mid-1990s to the present have delineated the pathway for parturition in this species, which again is distinct from human parturition in endocrine regulation characteristics.[6,7] With current advances in genomics and high-dimensional gene expression, protein, and metabolite profiling, greater attention has been given to studying humans and nonhuman primates.

Nonetheless, key conserved events in the parturition process are present across viviparous species. Pivotal among these are (1) transformation of the uterine smooth muscle (myometrium) from a quiescent to a highly excitable state, wherein it contracts forcefully and rhythmically to become the engine for birth; (2) remodeling of the extracellular matrix (ECM) of the cervical stroma such that the walls of the cervix distend in response to the contractions of labor to open the cervical canal and allow the conceptus to be moved into the vagina; and (3) weakening and rupture of the fetal membranes (the amnion and chorion) in response to paracrine interactions initiated by inflammation in the adjacent decidua (the endometrium of pregnancy). The synchronization of these events, referred to as active labor, occurs over a relatively short time and usually at term, which is the

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optimal time for birth considering adequate fetal maturity for survival outside the uterus and maternal physical, metabolic, and behavioral constraints for birthing and sustaining the neonate. The physiology and pathophysiology of human parturition will be considered from the perspective of the key functional components (i.e., the myometrium, cervix, decidua, and fetal membranes) of the gravid uterus and how their activities are controlled and coordinated to maintain pregnancy, facilitate birth, and nurture the neonate.

The gravid uterus is controlled by hormones generally classified as uterotropins and uterotonins. Uterotropins (e.g., estrogens and progesterone) establish the phenotype of the uterus by affecting its overall growth, contractile capacity, biomechanical integrity, and inflammatory status. Uterotonins (e.g., prostaglandins (PGs), which are 20-carbon unsaturated fatty acids that exert a spectrum of signaling effects on inflammation, smooth muscle function, and cellular metabolism[8–11]; and oxytocin (OT)) are hormones that directly stimulate or repress myometrial contraction and play critical roles at parturition by inducing the contractions of labor and cervical dilation. For most of pregnancy, pro-gestation uterotropins stimulate the growth of the uterus needed to accommodate the conceptus, and promote myometrial quiescence, closure of the cervix, and inflammatory quiescence at the maternal–fetal interface between the decidua and the fetal membranes. For parturition to occur, the effects of pro-gestation uterotropins (especially progesterone) diminish and the uterus is transformed to the laboring state through the combined effects of pro-labor uterotropins (mainly estrogens and PGs) and stimulatory uterotonins, especially prostaglandin F2 alpha ($PGF_{2\alpha}$), prostaglandin E2 (PGE_2), and OT. During this process, the myometrium becomes highly contractile and excitable to produce the coordinated and forceful contractions of labor in response to $PGF_{2\alpha}$ and OT. At the same time, the ECM of the cervix remodels in response to PGE_2, causing the tissue to become thin, soft, and distensible such that it dilates in response to increased intrauterine pressure induced by each contraction. Eventually, the cervix dilates wide enough to allow passage of the conceptus into the birth canal. Biochemical changes in ECM also occur at the fetal–maternal interface, due to inflammation in the decidua, to cause weakening and rupture of the adjacent fetal membranes (the amnion and chorion). Inflammation in the myometrium, cervix, and decidua is also a major component of the parturition processes and precedes the onset of active labor. The complex interplay between specific uterotropins, uterotonins, and proinflammatory hormones in the myometrium, cervix, and decidua that induce and mediate parturition will be discussed in this chapter.

The success of pregnancy is dependent on the appropriate timing of parturition. Ideally, birth should occur at a stage in gestation when the fetus is physiologically prepared for life outside of the uterus. In addition, specific physiological and behavioral processes (e.g., lactation) of caregivers are functional to ensure neonatal survival. Through natural selection, term gestation would have adapted in response to specific selective pressures in each viviparous lineage to maximize reproductive efficiency within the context of each species' overall reproductive strategy and environmental niche. This evolutionary perspective explains the remarkable diversity in parturition timing among viviparous species. Full term for human pregnancy is at the 40th completed week of gestation (calculated from the first day of the mother's last menstrual period). At this time, the probability of the fetus and mother surviving the parturition process is at its apex, with the risk for neonatal and/or maternal morbidity and mortality increasing exponentially the further from term that birth occurs (whether preterm or postterm). It is hypothesized that the extant timing mechanism for human parturition that causes term to occur at around the 40th week evolved through natural selection acting in response to a specific set of traits unique to the hominid lineage, the most influential of which is thought to be encephalization (a large brain). The fossil record shows a clear increase in brain and head size in progressive hominid species and reflects the potent selective pressure of encephalization. Herein, we explore how the physiology of human parturition was shaped by our unique natural history.

HUMAN EVOLUTION AND BIRTH TIMING

Perhaps no greater evolutionary selective pressure exists than to optimize reproductive outcomes by enhancing both maternal and fetal survival during parturition. The solution to optimizing reproductive fitness integrates multiple factors, including maternal habitus, fetal size, litter size, uterine shape, nutrient access, and other influences specific to each viviparous species. Thus, the mechanisms that promote reproductive fitness are likely to be unique to each organism. As completely sequenced genomes for the mouse, chimpanzee, and human became available, consistent with the notion of evolutionary pressure shaping pregnancy outcomes, genes involved in reproduction were among the most divergent groups.[12,13]

For human pregnancy, relatively recent evolutionarily events have likely impacted birth timing. According to the current fossil record, hominid brain size, and therefore head size, began to increase around 1 million years ago and has remained constant for at least the last 100,000 years.[14–17] This was, in part, mediated by an increase in the rate of brain growth during

fetal development that would have caused a progressive increase in the size of the fetal head at term. The large fetal head size at term poses two potential problems for infant and maternal survival. First, the size of the fetal head could reach the limit imposed by the pelvis, and, therefore, the advantages of encephalization would have been negated by the costs of neonatal and maternal loss due to difficulties at birth (Figure 42.1). The passage of the fetal head is further compromised by a relatively narrow maternal pelvis due to constraints that otherwise facilitate bipedalism. This relationship, the "obstetrical dilemma," results in significantly less obstetric capacity, that is, space between the infant head and maternal pelvis, in humans as compared to our nearest evolutionary relative, the chimpanzee.[15] Second, the large and rapidly growing human fetal brain consumes substantial energy resources from the mother. An alternative model to the relative size of the "obstetrical dilemma" is the "metabolic crossover hypothesis"[18] or the "energetics of gestation and growth hypothesis,"[19] which posits that the inability to provide adequate energy to the developing fetus results in "starvation" signals that initiate a cascade of events that ultimately culminates in labor and delivery.

The fact that encephalization continued in hominids suggests that natural selection solved the problem of cephalo-pelvic disproportion at term. One possible solution was to select for birth-timing mechanisms that cause the fetus to be born before its head becomes larger than the pelvic outlet. Indeed, human brain size at birth is less than 30% of its final adult size, while chimpanzee brain size is 50% of adult size.[20,21] The less developed human brain at birth could reflect relative changes in the timing for brain growth during development, a relative shortening of human gestation prior to parturition, or both. Recent allometric scaling and character tracing studies analyzing the ratio of neonatal brain or body size in comparison to the length of gestation indeed suggest that human gestation has been shortened relative to that of other primates.[22] One prediction of a relatively short human gestation is that the human neonate would be less mature at birth than other primates. This prediction is clearly evident. Human infants are cognitively, sensorily, and motorically less developed than neonatal chimpanzees and other primates,[23–25] a pattern described as secondary altriciality, due to the relative immaturity in the context of a long gestation in absolute time. The extant mechanism for human birth timing, however, is not ideal, as reflected by the difficulty of human parturition due mainly to the large fetal head and the relatively high incidence of pregnancy failure due to preterm or postterm birth. As natural selection operates at the population level over multiple generations, it could be argued that the present physiology

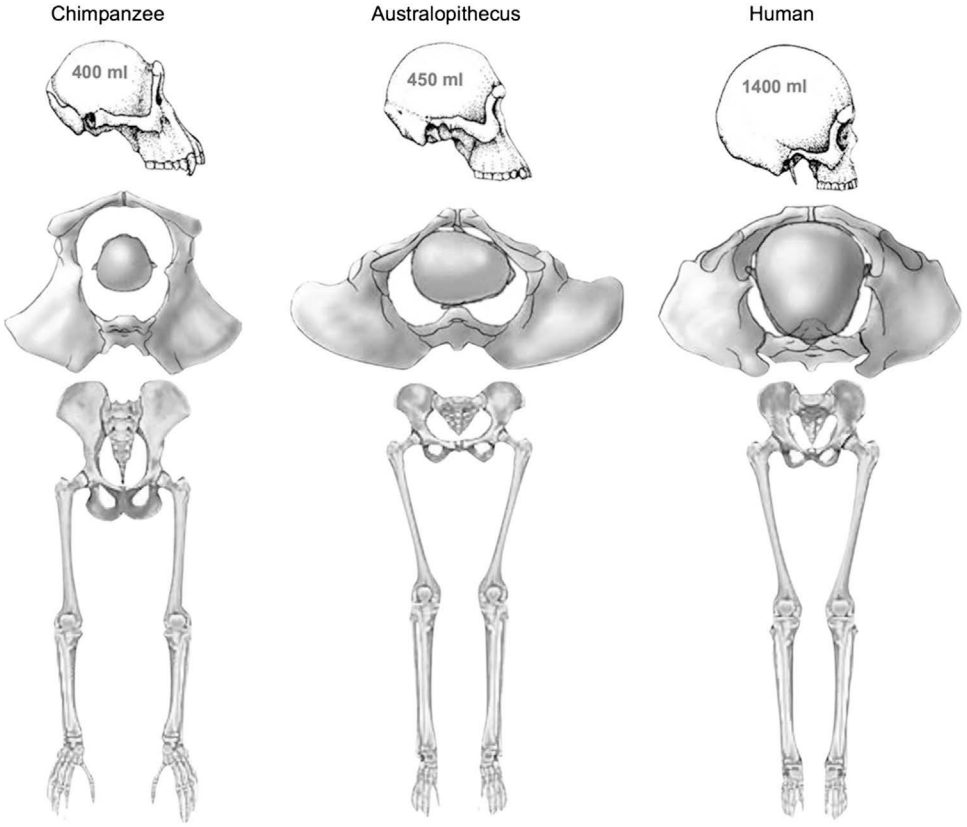

FIGURE 42.1 Changes in the cephalo-pelvic ratio through primate evolution. Relationship between the pelvic anatomy and the size of the fetal head at term in the human and chimpanzee, and an estimate of the cephalo-pelvic relationship of Australopithecus (2–4 million years ago) derived from the fossil record. *Source: Adapted from Rosenberg and Trevathan.*[15]

exists because the overall benefits of a birth-timing mechanism that favors encephalization outweigh the costs of reproductive failure (i.e., neonatal and maternal loss) due to preterm and postterm parturition. Whether preterm birth itself is uniquely human is also an area of current investigation. A potential corollary of the overall shortening of human gestation at term is that human pregnancy would be predisposed to terminate prematurely because of recent genomic changes that on average improve outcomes.[22,26] One recent study on captive chimpanzees suggests that the potential for infection-related preterm birth may have arisen prior to the human–chimpanzee evolutionary divergence,[27] but evidence of spontaneous preterm birth for chimpanzees in the wild has not been obtained. Interestingly, because of differences in pelvic shape, Neanderthal mothers did not have as severe constraints on delivery of the fetal head at term despite having the same fetal head size as anatomically modern *Homo sapiens*.[21,28] These considerations, more than of historical interest, foster new approaches for elucidating mechanisms for birth timing. Using comparative genomics across species, the signature for rapid evolution of genes specifically in the human genome can be used as a method to identify new components of the pathway leading to parturition.[22,29] Indeed, many of the established components of pregnancy maintenance and parturition, such as the progesterone receptor (PR), the estrogen receptor (ER), matrix metalloproteinase 8 (MMP8), the OT–neurophysin I precursor, and the prostaglandin E receptor 4 (PGEP4) receptor, show rapid evolution in their coding regions in humans.[22,30]

THE GRAVID UTERUS

The uterus is the principal organ of pregnancy and parturition. In the nongravid state and during most of the first trimester of pregnancy, the human uterus is a hollow, pear-shaped, muscular sack connected at its base to the upper end of the vaginal vault by the cervix, the narrow cylindrical portion of the lower uterine segment. The cervical cavity connects the uterine and vaginal cavities, and for most of pregnancy is blocked by a mucus plug that separates the sterile intrauterine environment from microorganisms in the vagina. The uterine wall consists mainly of smooth muscle cells to form the thick myometrium, which is the principal site of uterine growth during pregnancy and at parturition becomes the engine for birth. The inner lining of the uterus comprises the decidua, derived from the endometrium through the process of decidualization (discussed further in this chapter). The decidua is in close physical proximity to the chorion and amnion and is the maternal component of the physical fetal–maternal interface. It persists throughout pregnancy to form the physical barrier between the fetal chorionic tissue and the mother and is shed with the placenta and fetal membranes at birth.

The Parturition Process

Pregnancy and parturition involve specific activities by the myometrium, cervix, decidua, and fetal membranes that can be divided into four phases.[31]

Phase 0—establishment and maintenance of pregnancy: Pregnancy is established when the blastocyst implants into the endometrial stroma. During this process, the endometrium undergoes a complex morphologic transformation known as decidualization to form the specialized endometrium of pregnancy known as the decidua (see the section The Decidua and Fetal Membranes section). The decidua forms a physical and biochemical barrier between the fetal and maternal tissues that facilitates maternal immune tolerance of the allogeneic fetal graft. During Phase 0, the myometrium is maintained in a relaxed and quiescence state and grows to accommodate the enlarging conceptus. At the same time, the cervix remains rigid and unyielding, and its canal is closed by a mucus plug. The regulatory processes occurring within the decidua and myometrium required for Phase 0 are each dependent on progesterone (as discussed in this chapter).

Phase 1—preparation for labor: This phase represents the beginning of parturition and may start weeks prior to the onset of active labor. It involves changes in the gene expression in myometrial and cervical cells that, respectively, increase myometrial contractility and cause the cervix to become soft and compliant. In addition, tissue-level inflammation, indicated by increased infiltration and activation of macrophages and neutrophils and increased production of proinflammatory cytokines, occurs in the myometrium, cervix, and decidua.

Phase 2—labor and delivery: Active labor is defined as repetitive forceful uterine contraction associated with progressive dilation of the uterine cervix and rupture of the fetal membranes, and it occurs as a direct consequence of phenotypic changes in the myometrium, cervix, and decidua induced during Phase 1. In addition, the myometrium and cervix are exposed to increasing amounts of PGs, especially $PGF_{2\alpha}$, due to tissue-level inflammation, and increased OT secreted from the decidua and maternal pituitary. The combined effects of increased myometrial contractile efficiency, excitability, and uterotonin exposure and responsiveness cause the contractions of labor. In the cervix, increased exposure to PGE_2 combined with progesterone withdrawal, increased estrogen responsiveness, and tissue-level inflammation cause remodeling of the ECM that softens the cervical wall, leading to dilation

and effacement in response to the contractions of labor. Eventually, the cervix dilates enough to allow the contractions of labor to force the conceptus into the vaginal canal. Inflammation in the decidua also causes ECM remodeling in the adjacent fetal tissues that weakens the membranes, leading to rupture in response to increased intrauterine pressure.

Phase 3—hemostasis and involution: Immediately after the uterine contents are expelled, the myometrium undergoes a sustained and forceful contraction (mainly in response to OT), which helps constrict the spiral arterioles to facilitate postpartum uterine hemostasis. This contraction is critical to avoid a potentially lethal postpartum hemorrhage through the vascular lakes once occupied by the placental villi. During the first 3–6 weeks after birth, the myometrium gradually reverts to the nongravid state, and the cervix remodels and reverts to the narrow and rigid state. The decline in estrogen and progesterone levels after birth causes myometrial cells to atrophy, the vasculature of the uterus to regress, and blood flow to the uterus to be reduced. Within 3 to 5 months, cyclic activity of the endometrium reestablishes and the uterus is prepared for another pregnancy.

Thus, the physiology of human parturition centers on the hormonal mechanisms that control the growth and contractile properties of the myometrium, the biomechanical integrity of the cervix, and the immunologic and hormonal activity of the decidua.

The Myometrium

The human myometrium is composed of specialized smooth muscle cells called myometrial cells, which are arranged in randomly oriented interconnected bundles within an ECM of collagen, elastin, proteoglycans, and adhesion molecules such as laminin and fibronectin. The myometrium accounts for the majority of the uterine mass, and the remarkable growth of the uterus during pregnancy is due mainly to hyperplasia and hypertrophy of myometrial cells. Animal studies suggest that growth of the myometrium during pregnancy occurs by myometrial cell hyperplasia during the first half of pregnancy, referred to as the proliferative phase, and then myometrial cell hypertrophy and ECM remodeling during the latter half of pregnancy, referred to as the synthetic phase.[32–36] The myometrium also is highly compliant during most of pregnancy, and late in gestation is markedly distended by the conceptus. Thus, by term, the gravid uterus is endowed with a large number of hypertrophied and distended myometrial smooth muscle cells with the potential of generating the force needed to expel the conceptus.

Growth of the gravid uterus is thought to be regulated by the combined uterotropic actions of progesterone and estrogens produced by the maternal ovary or placenta, depending upon the time in gestation and the species, and locally produced growth factors such as epidermal growth factor and insulin-like growth factor I.[37,38] Growth of the myometrium can also be affected by mechanical distention. During the third trimester, the rate of fetal growth exceeds that of the uterus, leading to increased stretch of the myometrium. Animal and cell culture experiments suggest that signaling pathways activated in response to stretch cause myometrial cells to undergo hypertrophy and produce a less compliant ECM.[39,40] Increased myometrial stretch late in pregnancy is also thought to contribute to the cohort of signals that trigger labor (as discussed in this chapter).

Myometrial cells contract by converting chemical energy from the hydrolysis of adenosine triphosphate (ATP) into mechanical energy via the formation of rapidly cycling cross-bridges between actin and myosin, causing the filaments to slide over each other and shorten the cell.[41,42] The process is triggered by increased levels of intracellular free calcium (Ca^{2+}). Ca^{2+} binds with and activates calmodulin, which in turn activates the myosin light-chain kinase (MLCK) enzyme. MLCK phosphorylates the light chain of myosin, leading to activation of its ATPase activity.[41,43] The subsequent hydrolysis of ATP by myosin ATPase provides the energy for actin–myosin cross-bridges to form and slide against each other. Thus, myometrial cell contractility is controlled by events that affect the level of intracellular free Ca^{2+}. This is achieved by electrical or humoral mechanism through voltage- and ligand-gated Ca^{2+} channels, respectively. Myometrium is an excitable tissue in which contraction can be induced by an action potential in response to membrane depolarization that opens voltage-gated Ca^{2+} channels, especially the L-type Ca^{2+} channel, leading to an influx of extracellular Ca^{2+} that increases intracellular free Ca^{2+} and triggers the contractile machinery.

Quiescence of the gravid uterus is dependent on the resting membrane potential of myometrial cells, which establishes basal myometrial cell excitability. This is affected by the large-conductance Ca^{2+}-activated potassium (K^+) channel (BK), which facilitates the passive efflux of K^+ down its electrochemical gradient, leading to cell membrane hyperpolarization and decreased excitability.[44–50] The BK channel is activated (opened) by a depolarization in membrane potential and by an increase in intracellular free Ca^{2+}[51,52] and plays a major role in the relaxation phase of a typical contraction. In myometrial cells, BK channels are activated by relaxatory uterotonins.[53,54] BK subunit expression is decreased in association with the onset of labor.[55] Pharmacologic blockade of the BK channel depolarizes myometrial cells, increases intracellular free Ca^{2+} levels, and increases the frequency and amplitude of phasic contractions.[44]

Humoral control of myometrial contraction is mediated by the activation of intracellular signaling

pathways that stimulate the release of Ca^{2+} from intracellular stores through ligand-gated Ca^{2+} channels in the sarcoplasmic reticulum and on the cell membrane. Stimulatory uterotonins such as OT and $PGF_{2\alpha}$ induce contraction by first activating cognate G protein–coupled receptors linked to Gαq (the alpha subunit of heterotrimeric G protein q). Gαq activates phospholipase C (PLC), which in turn catalyzes the formations inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG).[56–59] IP3 binds to the ligand-gated Ca^{2+} channel to induce the release of stored Ca^{2+} from the sarcoplasmic reticulum, and DAG activates protein kinase C (PKC), which augments L-type Ca^{2+} channel activity.[58–61] The net effect is a sustained increase in intracellular free Ca^{2+}.

Relaxatory uterotonins (e.g., PGI_2, beta-adrenergic receptor activators, and nitric oxide (NO)) suppress contractile activity by inhibiting Ca^{2+} mobilization and inhibiting the activity of specific kinases in the contractile apparatus.[62] These effects are mediated mainly by the cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) signaling pathway. In general, relaxatory uterotonins bind G protein–coupled receptors linked to $G\alpha_s$ (the G_s alpha subunit). $G\alpha_s$ activates membrane-bound adenylate cyclase, which catalyzes the formation of cAMP. cAMP activates PKA, which decreases intracellular Ca^{2+} mobilization by inhibiting IP3-induced release of Ca^{2+} from the sarcoplasmic reticulum, inhibiting L-type Ca^{2+} channel activity, and stimulating the sequestration of Ca^{2+} into intracellular stores. PKA also inactivates MLCK and PLC and stimulates BK channel expression. Thus, the cAMP–PKA signaling pathway exerts potent relaxatory effects on the myometrium. Myometrial cell relaxation also is induced by NO, a highly diffusible signaling molecule involved in many physiological and pathological processes.[63–67] NO activates soluble guanylate cyclase, which catalyzes the formation of cyclic guanosine monophosphate (cGMP). cGMP activates PKG, which functions in a similar way as PKA to repress intracellular free Ca^{2+} mobilization and the activity of MLCK and PLC. cGMP causes relaxation in vascular smooth muscle, but it is highly inefficient in the myometrium, and there is some controversy as to its role in maintaining uterine relaxation and mediating relaxatory actions of NO in myometrial cells.[68] NO is produced by the conversion of L-arginine into L-citrulline and NO, which is catalyzed by various isoforms of the nitric oxide synthase (NOS) enzyme. In the human uterus, NOS protein and mRNA are low and not correlated with the stage of gestation or the onset of labor, suggesting that endogenously produced NO is not a modulator of myometrial contractility in human parturition.[69]

A novel, recently described mechanism for maintenance of uterine quiescence involves progesterone-mediated induction of caspase-3 activity.[70] In the uterine myocyte, active caspase-3 degrades contractile proteins. Decreased progesterone signaling late in gestation reduces anticontractile caspase-3 actions and may promote labor. Interestingly, the myocyte, while inducing caspase-3 action, which is normally apoptosis promoting, simultaneously elevates antiapoptotic pathways to avoid myocyte cell death.[71,72]

The myometrium lacks neural connections, and an electrical pacemaker system has not been identified in the uterus. Nonetheless, actions potentials propagate between neighboring myometrial cells and myometrial cell bundles to synchronize contractions temporally and spatially across the entire uterus. Contraction synchrony is achieved by intercellular communication through specialized channels, known as gap junctions, that facilitate electrical and metabolic coupling between adjacent myometrial cells.[73] Small molecules, including ions, amino acids, and second messengers, flow between cells through gap junctions, and the development of spatially and temporally coordinated Ca^{2+} waves can also be mediated through gap junctions.[74] For most of human pregnancy, gap junctions between myometrial cells are scant, and the muscle produces only weak and uncoordinated contractile activity.[75,76] Late in gestation and in association with the parturition process, expression of gap junction alpha-1 (GJA1; also known as connexin 43) increases in human myometrium, leading to an increase in the number of gap junctions between myometrial cells, and this coincides with the development of the coordinated forceful contractions of labor.[73,76–81] In mice, ablation of GJA1 expression in the myometrium disrupts parturition and prolongs pregnancy.[82,83] Expression of genes encoding gap junction subunits and the assembly of functional gap junctions in myometrial cells is increased by estrogens and PGs and decreased by progesterone.[84,85]

Thus, myometrial contractility is controlled by processes that affect (1) intracellular free Ca^{2+} levels; (2) electrical excitability, determined by the resting membrane potential; (3) responsiveness to uterotonic hormones mediated by the level of cognate receptors and the activity of associated signaling cascades that affect Ca^{2+} mobilization and myosin ATPase activity; and (4) the extent of gap junction electrical and biochemical coupling of myometrial cells and myometrial bundles. These processes are affected by uterotonic and uterotropic hormones.

The two contractile states of the gravid uterus, quiescence and laboring, are established by the combined actions of uterotropic and uterotonic hormones (Figure 42.2). Uterotropic hormones, especially progesterone and estradiol, affect contractile capacity by modulating the expression of genes in myometrial cells that encode factors collectively referred to as contraction-associated proteins (CAPs). Some important

CAPs include receptors for uterotonic hormones (e.g., OT receptor (OTR), $PGF_{2\alpha}$ receptor (FP), and PGI_2 receptor (IP)); enzymes involved in PG biosynthesis (PG endoperoxidase type 2 (PTGS2)) and metabolism (15-hydroxyprostaglandin dehydrogenase (HPGD)); ion channels, especially the L-type Ca^{2+} channel and the BK channel; and gap junction proteins, especially GJA1.

For most of pregnancy, the myometrium is maintained in a quiescent state (i.e., Phase 0) due to the relaxatory actions of progesterone. In addition, the myometrium undergoes remarkable growth to accommodate the developing conceptus and prepare for its role as the engine for birth at the end of pregnancy. Proliferation and hypertrophy of myometrial cells during Phase 0 are thought to be induced by the combined effects of estrogens (estradiol, estrone, and estriol) and uterine wall distention imposed by the growing conceptus. Estrogens also promote expression of the type B progesterone receptor (PR-B), which mediates the relaxatory actions of progesterone.[86] Thus, for most of pregnancy, the net effects of progesterone and estrogens are myometrial quiescence and growth.

Weak and uncoordinated contractile activity, known clinically as Braxton-Hicks contractions or contractures, occur during Phase 0 and may increase in frequency and intensity near term. Contractures are not powerful enough to move the conceptus or dilate the cervix, and likely reflect basal excitability of the myometrium in response to increased distention. The switch in myometrial contractility from contractures to contractions is a hallmark of Phase 1 and is due to a phenotypic change in the contractile capacity of the myometrium in preparation for the onset of active labor (i.e., Phase 2).

In all species, the transition from Phase 0 to Phase 1 can be induced by progesterone withdrawal (as discussed in this chapter). In this regard, two functions of progesterone in the physiology of pregnancy become critical: (1) the mechanism by which it promotes myometrial uterine quiescence, and (2) the mechanism by which its withdrawal induces labor. The general consensus is that progesterone promotes myometrial relaxation by

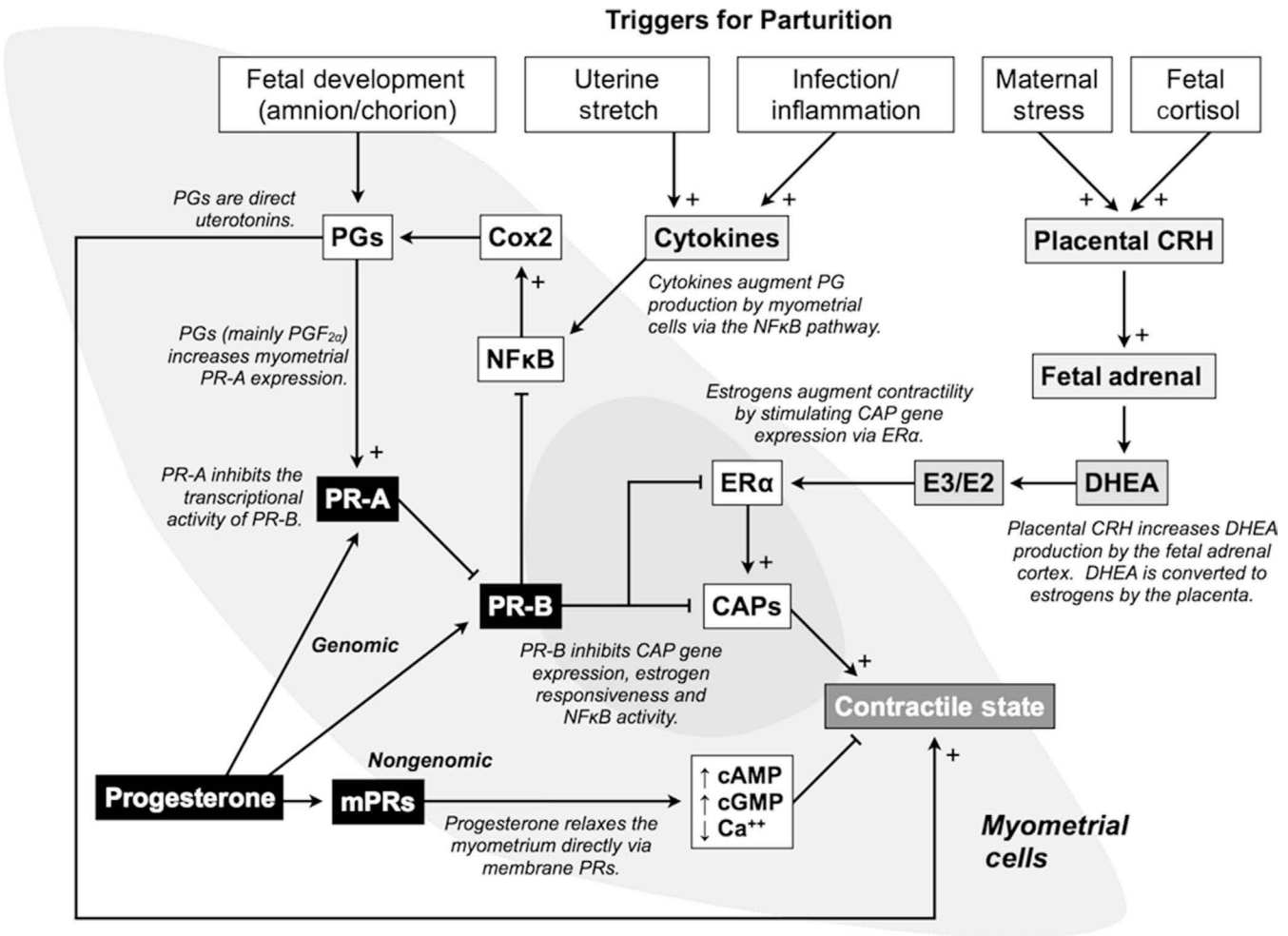

FIGURE 42.2 Hormonal pathways regulating myometrial contractility.

inhibiting the expression of genes encoding pro-labor stimulatory CAPs. It also blocks the capacity for estrogens to increase expression of stimulatory CAPs by inhibiting expression of estrogen receptor alpha (ERα). The assumption is that progesterone specifically blocks the pro-labor actions of estrogens but does not affect the pro-growth actions of estrogens. This may be because the pro-growth actions of estrogens on the pregnancy myometrium are mediated via an extranuclear mode of ERα action, whereby ERα interacts with cytoplasmic signaling molecules to initiate the mitogen-activated protein kinase (MAPK) pathways that augment cell proliferation and hypertrophy.[87] Upon progesterone withdrawal, genomic actions of ERα are activated to augment expression of stimulatory CAP genes, leading to increased myometrial excitability and contractility. Thus, the switch to Phase 1 and eventually Phase 2 is mediated by the combined and functionally linked effects of progesterone withdrawal and estrogen activation.

The phenotypic transition to the laboring state also involves increased myometrial excitability due to relative myometrial cell depolarization, increased sensitivity to uterotonins due to increased myometrial cell expression of cognate receptors (especially OTR and FP), increased local PG production due to tissue-level inflammation and increased expression of PG synthetic enzymes (especially PTGS2), and increased electrical coupling between myometrial cells due to increased gap junction formation as a result of increased GJA1 expression. Thus, the quiescent state is actively achieved by the dominant relaxatory actions of progesterone and tropic actions of estrogens, and transition to the laboring state is caused by progesterone withdrawal and estrogen activation and increased responsiveness to stimulatory uterotonins. The mechanism of action of key uterotropins and uterotonins in the parturition process is discussed further in this chapter.

The Cervix

The cervix is the cylindrical extension of the lower uterine segment that connects the uterus with the upper end of the vagina (for reviews, see Refs 88,89). It contains a central canal that links the uterine and vaginal cavities, and as such serves as the gateway between the uterus and the vagina. In the nonpregnant state, the cervical canal is narrow and open to allow menstrual discharge and entry of sperm. During most of pregnancy, the cervical canal remains narrow (i.e., competent) to contain the conceptus in the uterus, and it is closed by a mucus plug to separate the uterine and vaginal cavities. For birth to occur, the cervical ECM is remodeled, causing the tissue to become soft and distensible. This causes the central canal to widen and allow the conceptus to pass into the vagina. Contractions of labor cannot produce birth if the cervix is rigid and closed, whereas an open cervix (i.e., cervical incompetency) can lead to birth even without myometrial contractions. Thus, the cervix serves a key mechanical role during pregnancy to either contain and protect the conceptus in the uterus or allow its passage into the vagina. The biomechanical integrity of the cervix is therefore crucial for the success of pregnancy, and its control is a key part of the parturition process.

Cervical anatomy can be divided into three components: (1) the ectocervix that projects into the vagina and is lined by epithelial cells, (2) the endocervix that forms the lining of the cervical canal, and (3) stromal fibroblasts that form the body of the cervix that connects to the myometrium. The main role of the endocervical epithelial lining is to prevent vaginal microorganisms from entering the uterine cavity. The main function of the stromal fibroblasts is to produce a collage-rich ECM that confers rigidity to regulate the size of the cervical canal.[88]

During pregnancy, endocervical epithelial cells proliferate and form endocervical glands and crypts that extend and branch deep into the fibrous connective tissue of the cervical wall. By late in the third trimester, endocervical glands occupy ~50% of the entire cervical mass. The glands secrete an antibacterial mucinous substance that plugs the cervical canal. The endocervical epithelial cells also secrete defensins and mediate inflammatory responses that inhibit bacterial proliferation. At certain stages of pregnancy, the cells produce high levels of PG synthetic enzymes,[90] and they secrete cytokines and chemokines (especially interleukin 8 (IL8))[91] and protease inhibitors[92–94] that affect the integrity of the stromal ECM and therefore the mechanical properties of the cervix. During the early stages of pregnancy, the volume of cervix occupied by endocervical glands increases and is directly associated with the length of the cervical canal.[95] After midgestation, the cervical gland area begins a gradual decline that correlates with progressive cervical softening and shortening of the cervical canal.

The cylindrical wall of the cervix is composed mainly of endocervical fibroblasts and a relatively low number (10–15%) of myometrial cells.[88,89] The endocervical fibroblasts produce a dense and rigid fibrous ECM composed of type I and type III collagen in conjunction with the structural proteins, elastin and fibronectin, hyaluronate, and various proteoglycans such as versican and decorin. The unyielding ECM keeps the cervical canal narrow for most of pregnancy by resisting tensile forces generated by the myometrial contractions that exert a circumferential upward pull on the cervical wall; increased intrauterine pressure, especially late in pregnancy when the conceptus enlarges and myometrial contractions become more frequent; and the general downward force of gravity on the uterine contents. ECM rigidity is determined by the orientation and length of collagen fibers. In general,

closely parallel collagen bundles with fiber lengths of at least 20 μm augment tissue rigidity, whereas short collagen fibers in a disarrayed configuration decrease tissue rigidity. Biomechanical properties of the cervix during human pregnancy can be divided into four phases: *softening, ripening, dilation, and repair*.[96]

Cervical softening: During the first few months of human pregnancy, the cervix is rigid and unyielding. Cervical rigidity, however, progressively decreases after midgestation without losing tensile strength. This process is mediated primarily by restructuring of the fibrillar collagen architecture. The process also involves increased vascularity and edema, hypertrophy of cervical stroma, and hypertrophy and hyperplasia of the cervical glands, and is generally related to cervical growth.

Softening of the cervical ECM causes gradual thinning of the cervical wall and shortening of the cervical canal (i.e., the distance between the uterine and vaginal openings). Late in the softening phase and usually near term, the cervical canal may dilate by up to 3 cm and the cervical plug may be lost. Although cervical softening does not compromise tensile strength, the structural changes can affect the timing of parturition. Ultrasonography monitoring of the cervix during pregnancy has shown that aberrant softening that excessively shortens or deforms the cervical canal is a strong predictor of preterm birth.[97] This may cause an untimely breach of the cervical barrier to opportunistic microbial invasion from the vagina to the amniotic cavity that triggers the parturition cascade and causes preterm birth.

Animal studies have indicated that relaxin produced by the placenta, chorion, and decidua is a major regulator of cervical softening.[98] Relaxin promotes cervical softening in rats, mice, and guinea pigs.[99–102] In relaxin-deficient mice, defects in cervical remodeling during pregnancy are associated with difficult parturition.[103] In the absence of relaxin, cervical softening during pregnancy is deficient but cervical growth is not affected.[104] Progesterone and estrogen appear to modulate the actions of relaxin on the pregnancy cervix, possibly by affecting relaxin receptor levels or signaling.[105]

Another factor that may regulate cervical softening is thrombospondin 2 (TSP2). Mice deficient in TSP2 have increased cervical softening at midgestation but normal parturition at term,[106] suggesting that, at least in mice, premature cervical softening does not affect the timing of parturition. This is in contrast to clinical observation showing that shortening of the cervix at 18–22 weeks of gestation, presumably due to excessive softening, is strongly predictive of preterm birth, especially if accompanied by funneling of the membranes.[107,108]

Thus, the general consensus is that gradual cervical softening with maintained structural integrity represents an adaptation of the cervix to pregnancy, and likely is part of the cervical growth process. This process appears to be independent of parturition-associated changes in the cervix that are needed for delivery of the conceptus. Although cervical softening may not be part of the parturition process per se, it may affect the timing of parturition by affecting susceptibility to intrauterine infection. Effects of softening during pregnancy on the mechanical and biological competence of the cervix are therefore critical factors in the trajectory toward parturition.

Cervical ripening: Cervical ripening occurs late in pregnancy and involves major changes in the structure and composition of the cervical ECM leading to a loss of structural integrity and tensile strength.[109,110] The process of cervical ripening is an early event in the parturition cascade and precedes the onset of active labor by several weeks. During ripening the cervix becomes soft, thin, and easily distensible, and its load-bearing capacity decreases. The dramatic biomechanical change is caused primarily by a decrease in the concentration and organization of collagen in the ECM. Modification of the collagen architecture is mediated by an increase in hydrophilic glycosaminoglycans, especially decorin, which binds to collagen fibers and disrupts fibrillar organization,[111,112] and increased expression of hyaluronate,[113–115] which weakens the interaction of collagen with fibronectin and increases the gap between collagen fibers by attracting water into the interfibrillar space, thereby increasing cervical hydration and collagen dispersal.[89,109,114–116] Water imbibition also increases collagen solubility and susceptibility to digestion by extracellular MMPs, especially collagenase. The net effect is increased dispersal and disorganization of shortened collagen fibers. As described in this chapter, short and disarrayed collagen fibers increase tissue softness, pliability, and distensiblity.

Progesterone promotes cervical competency and prevents ripening. Various mechanisms for the effect have been identified. Progesterone inhibits collagen breakdown by increasing the expression of tissue inhibitor of metalloproteinase 2 (TIMP2) and inhibiting the expression and activity of MMPs by endocervical fibroblasts.[117–120] In addition, progesterone antagonizes estrogen-induced collagenase expression by endocervical fibroblasts.[121,122] Progesterone also inhibits hyaluronate synthesis in human endocervical fibroblasts by inhibiting expression of the hyaluronan synthase 2 gene.[123,124] A decrease in hyaluronate would prevent water imbibition and collagen dissolution and therefore maintain ECM rigidity. Consistent with progesterone withdrawal at parturition, hyaluronate levels in the cervix increase prior to the onset of parturition,[125] possibly due to the combined effects of progesterone withdrawal and stimulation by estrogens and PGs. In all species studied so far, treatment with PR antagonists at any stage of pregnancy promotes cervical ripening.[126–130] Sensitivity

of the cervix to RU486 (mifepristone)-induced ripening increases as pregnancy nears term, suggesting that the capacity for progesterone to maintain cervical competence decreases as pregnancy progresses.[131]

Cervical ripening is an inflammatory process that involves infiltration of activated myeloid cells into the cervical stroma.[132–135] Induction of tissue-level inflammation in the cervix is thought to initiate a positive-feedback cycle that augments the inflammatory state, leading to increased accumulation of ECM degradative activity. Progesterone is thought to counteract that process by inhibiting the responsiveness of endocervical fibroblasts to proinflammatory cytokines.[136]

PGs are important regulators of parturition (as discussed in this chapter) and play a pivotal role in cervical ripening. Clinical ripening of the cervix leading to the induction of labor is routinely performed by treatment with PGE_2 or synthetic analogs (e.g., misoprostol) administered locally (intracervically or vaginally) or intravenously (IV).[137–141] Based on ECM composition, PGE_2-induced ripening is indistinguishable from spontaneous ripening and is effective at all stages of pregnancy.[142] PGE_2 ripens the cervix, at least in part by modulating glycosaminoglycan expression by endocervical fibroblasts via its interaction with the type 4 PGE2 receptor.[143,144]

Estrogenic actions in the cervix are also thought to promote ripening by augmenting collagenase expression in endocervical cells.[121,122] Estrogens also increase mucus secretion by endocervical epithelial cells and promote the formation of endocervical glands and cervical growth.[145] These pro-gestational actions are likely due to the modulation of estrogenic actions on the cervix by progesterone. Upon progesterone withdrawal, however, estrogenic effects on the cervix promote ripening via increased collagenase expression.

Cervical dilation: Cervical dilation occurs at the time of active labor and involves dramatic thinning (effacement) of the cervical wall such that it virtually dissolves into the lower uterine segment. At this stage, the cervical resistance to tensile force collapses, and in response to the contractions of labor and pressure from the presenting fetal part (usually the head), the cervix dilates to open the central canal and allow passage of the conceptus into the vagina.

Dilation is associated with increased expression and tissue-level activity of MMPs, especially collagenase. Dilation also involves a massive influx of leukocytes into the cervical stroma, and these inflammatory cells are a major source of collagenase.[146] Immune cell infiltration is promoted by the potent chemokine IL8, whose levels increase dramatically in the cervix of women during labor and after delivery.[147] Cervical dilation also involves increased production of hyaluronate in response to progesterone withdrawal. Hyaluronate softens the cervical ECM and augments tissue-level inflammation.[123,125]

Cervical repair: Repair and recovery of the cervix back to the competent state occur in the immediate postpartum period. This process involves the inhibition of proinflammatory pathways, cessation of collagen dissolution, and promotion of collagen synthesis and reestablishment of the collagen architecture to confer rigidity. Relief of mechanical stretch also may contribute to inhibiting proinflammatory signaling pathways. The hormonal control of cervical repair and recovery is not clearly understood, but it is likely to involve local paracrine interactions that inhibit inflammatory pathways and the re-establishment of cycling estrogenic and progesterone effects on cervical growth.

The Decidua and Fetal Membranes

The fetal membranes, composed of the amnion and chorion, together with adjacent decidua of maternal origin, provide a dynamic site for regulation of both myometrial activity and fetal maturation. Essential functions for these tissues include paracrine signaling between the mother and fetus to initially limit the potential for the onset of the characteristic changes associated with the onset of labor before term, a barrier function to retain amniotic fluid and prevent invasion of the fetal compartment with circulating biochemical signals and potential microbial pathogens, and, finally, the key to the parturition process, controlled membrane rupture to promote efficient expulsion of the fetus and placenta.[148,149]

The structure of the fetal membranes at term arises as the result of sequential morphological events that begin at implantation. Embryo implantation occurs as the blastocyst transverses a disrupted endometrial cell surface, such that the blastocyst occupies an interstitial location in the uterus.[150] The endometrium that comes to surround the implanted blastocyst forms the early decidua. The decidua is initially regionalized as the decidua basalis, between the blastocyst and the myometrium, and the decidua capsularis, filling the overlying area through which the blastocyst penetrated the uterine wall.[151] The endometrial lining on the uterine surface opposite the implantation site is designated the decidua vera. In human pregnancy, the expansion of the conceptus leads to fusion of the amnion and chorion with the decidua vera, and the decidua capsularis degenerates.[150] The cytotrophoblast cells of the chorion directly contact the maternal decidual cells (Figure 42.3). While initially filling only a portion of the chorionic cavity, the amnion expands to enclose the developing embryo and physically oppose the chorion by the 12th week of human gestation. The chorion and amnion, while opposed to one another through pregnancy, remain separable layers that allow distinct patterns of disruption during membrane rupture, as is described in greater detail in the discussion on membrane rupture in this section.[152] Interestingly, the

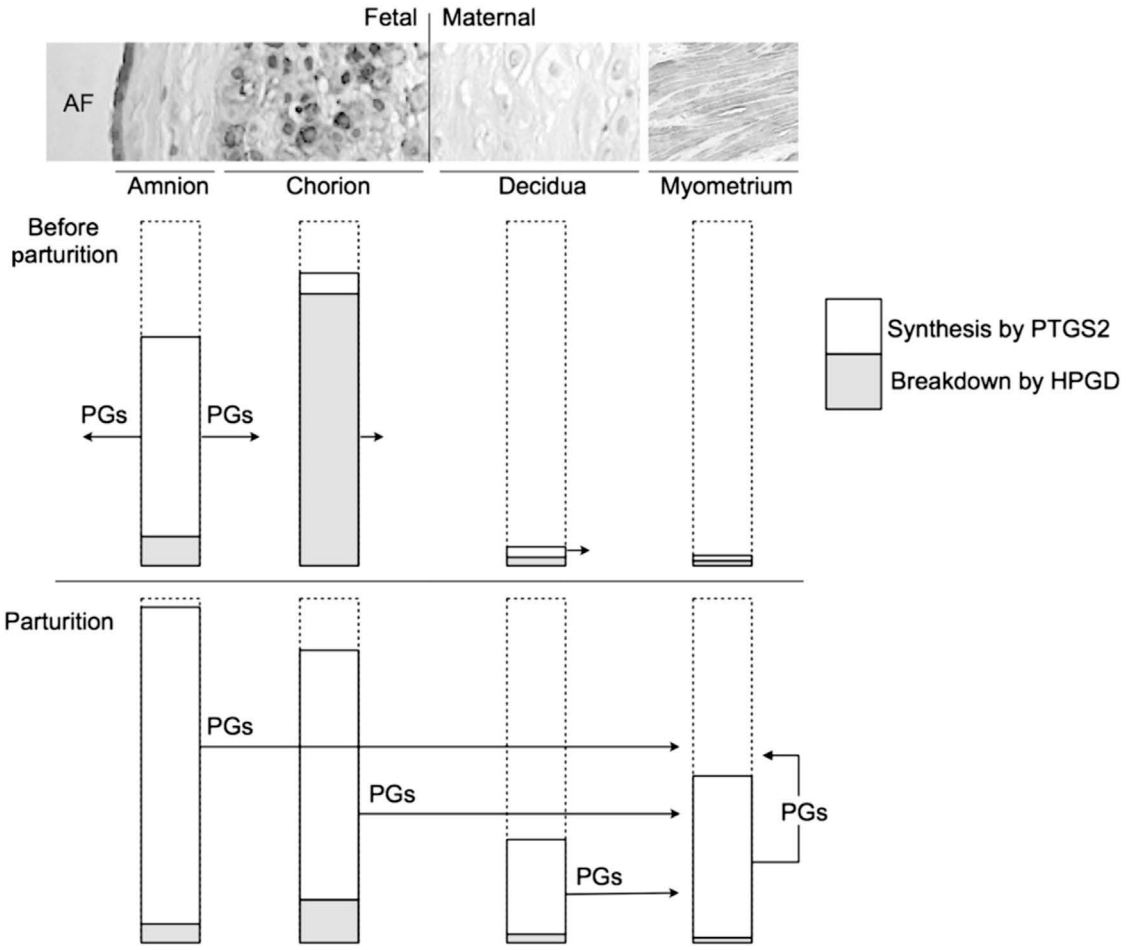

FIGURE 42.3 Changes in PG–endoperoxide synthase 2 (PTGS2) and hydroxyprostaglandin dehydrogenase (HPGD) activities in the gestational tissues (amnion, chorion, decidua, and myometrium) associated with the onset of human labor. Parturition is associated with increased PTGS2 activity in all tissues. HPGD is predominantly expressed by the chorion and effectively blocks amnion PGs from accessing the myometrium. Parturition is associated with decreased HPGD activity in the chorion that could allow more active PG to reach the myometrium. PGs are also produced by the myometrium at parturition and likely have autocrine and paracrine effects on contractility.

human fetal membranes are not vascularized. This lack of vascularity limits the ability of the fetal membranes to transfer nutrients between the mother and the fetus.

Paracrine signaling and biochemical functions of the fetal membranes: Through an extensive series of human, nonhuman primate, and other mammalian studies, the critical roles for the amnion and chorion in generating the biochemical signals that restrain or accelerate parturition, and promote fetal organ maturation, have emerged.[148] These critical biochemical pathways center on PG and glucocorticoid biosynthesis and degradation.

Prostaglandin metabolism: The amnion and chorion both contain the enzymatic machinery for PG biosynthesis.[153–155] In contrast, degradation of bioactive PGs to inactive metabolites is restricted to the cytotrophoblasts of the chorion.[156–158] This anatomical localization of the primary PG-degrading enzyme 15-hydroxyprostaglandin dehydrogenase (HPGD) provides an effective barrier to the passage of PGs (especially PGE_2 and $PGF_{2\alpha}$) that promote myometrial contraction and cervical ripening from the membranes to the uterus prior to term.[159]

PG biosynthesis in the fetal membranes occurs most robustly in the amnion and, to a lesser extent, in the chorion. The primary cell type within the amnion generating PGE_2 is the fibroblast.[160] Amnion-derived PGE_2 occurs largely through the initial step of type IV $cPLA_2$ releasing arachidonic acid from membrane phospholipids.[161] Arachidonic acid serves as the substrate for PG–endoperoxide synthases 1 (PTGS1, aka cyclooxygenase 1, or COX1) and PTGS2 (aka COX2) to generate PGH_2 (Figure 42.4), as described in the "Prostaglandins" section of this chapter. PGH_2 is an unstable intermediate that undergoes conversion to mature prostaglandin types (e.g., PGE_2, $PGF_{2\alpha}$, thromboxane, and prostacyclin) by specific synthases, and is capable of acting on their cognate G protein–coupled receptors.[162] With term and preterm labor in humans, the production of PGs by the

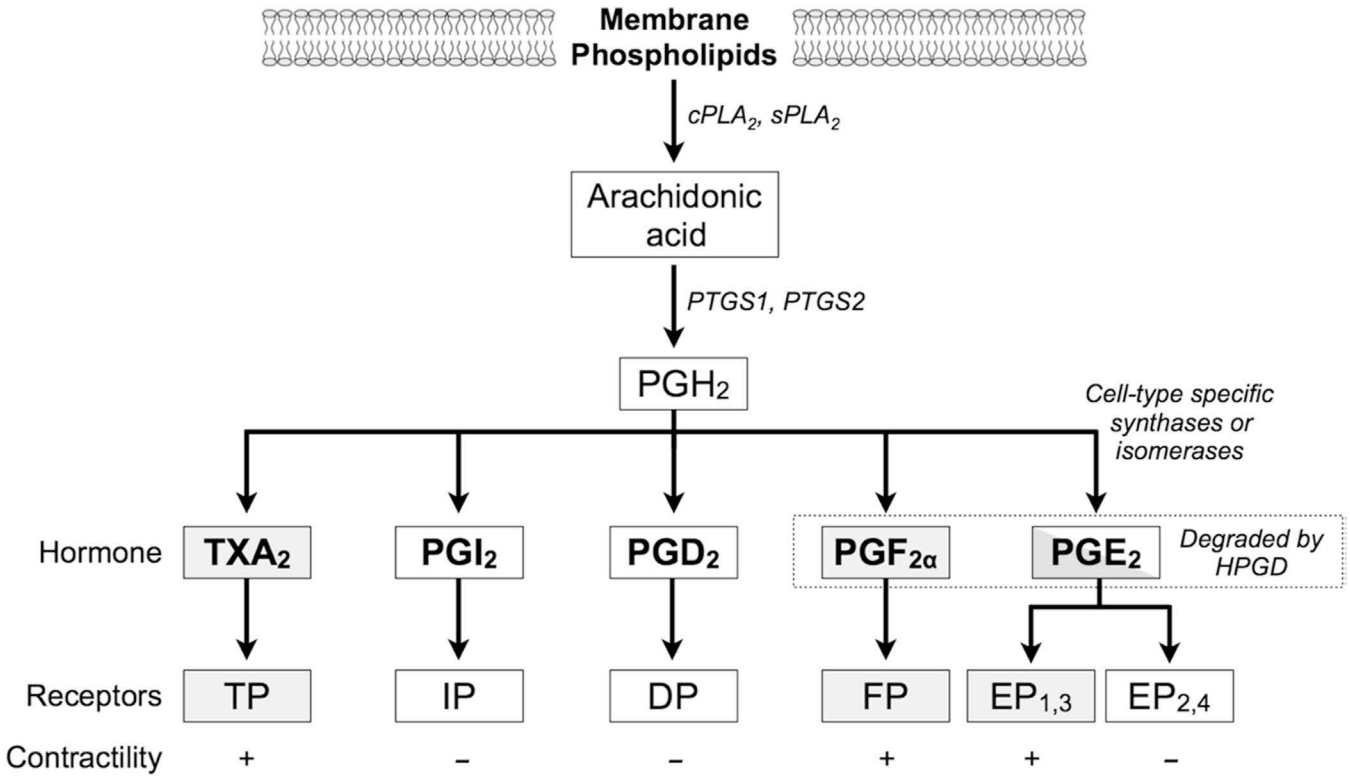

FIGURE 42.4 Synthesis of biologically active PGs from membrane phospholipids, the key enzymes involved, the receptors with which they interact, and their effects on myometrial contractility.

amnion is significantly increased.[163,164] The primary PG produced is PGE_2, which is capable of both stimulating uterine contractions, via EP1 and EP3 receptors (prostaglandin E receptor 1, subtypes EP1 and EP3) on myometrial cells, and cervical ripening.[165,166] This induction of PGE_2 is associated with enhanced expression of PTGS2 in the amnion.[160,167] PTGS2 co-localizes in expression with microsomal PGE synthase 1, which is inducible by cytokines or endotoxin,[168] and also couples its activity with microsomal PGE synthase 2, which is constitutively expressed in the Golgi apparatus.[169,170] Interestingly, glucocorticoids, which are increased in production in both maternal and fetal compartments at term or during stress throughout gestation, lead to the induction of PTGS2 and PGE_2 synthesis from primary amnion cells, in marked contrast to the usual antiinflammatory actions of glucocorticoids through the type II glucocorticoid receptor (NR3C1).[171,172] As the fetal membranes also have the ability to regenerate cortisol, a positive-feedback network accelerating contraction-promoting PGs has been proposed.[173,174]

PG degradation largely occurs through the action of HPGD for those types most highly implicated in the onset of labor, PGE_2 and $PGF_{2\alpha}$. Prostacyclin, thromboxane A_2, and prostaglandin D_2 (PGD_2), in contrast, undergo spontaneous degradation with a very short half-life.[175] Several stimuli modulate HPGD expression and activity in a manner consistent with its postulated role in restraining the onset of labor. Prior to term, progesterone promotes HPGD activity.[176] At term, HPGD activity normally decreases.[177] Cortisol, which increases at term, inhibits HPGD activity in vitro and may contribute to this decline.[178–180] In contrast, corticotropin-releasing hormone (CRH), which also increases as term approaches, stimulates HPGD activity in chorionic trophoblasts.[181] In women with chorioamnionitis and infection-associated preterm labor, the number of HPGD-expressing chorionic trophoblasts was found to be reduced, suggesting increased potential for passage of labor-augmenting PGE_2 to the myometrium.[157]

Glucocorticoid metabolism: Glucocorticoids, derived from the maternal and (later in gestation) fetal adrenals, play critical roles in accelerating fetal organ maturation (skin, gastrointestinal tract, and lung, among others) necessary for ex utero survival.[182–185] The primary glucocorticoids affecting development are cortisol in humans and other species with 17α-hydroxylase activity in the adrenal zona fasciculata, and corticosterone in those species lacking 17α-hydroxylase activity in the adrenal (e.g., rodents). In most species, maternal and fetal serum glucocorticoid concentrations increase as gestation progresses[186–188]; for sheep, this glucocorticoid rise is the trigger to initiate parturition by virtue of its actions in promoting estradiol synthesis from progesterone in the

placenta.[3,4] Conversely, glucocorticoids, when present in excess, exhibit teratogenic effects, including apoptosis in the fetal brain.[189–191] As a consequence, complex mechanisms have evolved to control glucocorticoid access to the fetal compartment.

The primary glucocorticoid-metabolizing enzyme for most of gestation in the fetus and placenta is 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2).[192,193] This NAD-dependent oxidase converts cortisol to the inactive metabolite cortisone, or corticosterone to its inactive metabolite, dehydrocorticosterone. The conversion to inactive metabolites limits the potential teratogenic actions of glucocorticoids on fetal tissues and other effects that accelerate labor, such as fetal membrane PG production. As a consequence of the high 11βHSD2 activity, human placenta and fetal blood display high concentrations of cortisone.[194,195] Even in the context of the robust 11βHSD2 activity, the cortisol–cortisone ratio in the amniotic fluid increases through gestation and is higher than that measured in cord blood.[196,197] This finding suggested that the fetal membranes could themselves be a site for cortisol synthesis. Indeed, studies have demonstrated that human amnion develops enhanced capacity to regenerate cortisol from cortisone as gestation progresses, potentially accounting for the elevated cortisol–cortisone ratio in amniotic fluid.[197,198] The regeneration of cortisol from cortisone is achieved through the activity of 11βHSD1, a nicotinamide adenine dinucleotide phosphate oxidase–dependent oxoreductase.[193,199] Expression of 11βHSD1 has been demonstrated in the amnion epithelium, fibroblasts, and chorionic trophoblasts, along with decidual stromal cells adherent to the chorion.[173,174] The expression of 11βHSD1 and its activity have been shown to increase through gestation. In contrast, little or no 11βHSD2 activity or mRNA expression has been detected in the amnion and chorion.[174]

Interactions of PG and glucocorticoid pathways to initiate labor: The predominant action of glucocorticoids on proinflammatory cytokines and PGs under nongravid conditions is to suppress their synthesis and downstream signaling through the inhibition of transcription factors such as NFκB (nuclear factor kappa light-chain enhancer of activated B cells), activating protein 1 (AP1), signal transducers and activators of transcription (STATs), and MAPKs.[200,201] Glucocorticoid action on the fetal membranes during gestation markedly diverges from this typical paradigm. In human fetal membrane primary cell cultures, glucocorticoids stimulate, rather than inhibit, PGE_2 synthesis.[160,202] This stimulatory effect is mediated by dose-dependent actions on amnion fibroblasts, which possess cytosolic phospholipases A2 ($cPLA_2$), PTGS2, and PGE synthases. As fetal membranes lack expression of PR-B but express PR-A, which is modulated by glucocorticoids, this paradoxical activity could occur either through the classical glucocorticoid receptor (GR) or through transdominant repression of glucocorticoid receptors by PR-A, as it is attenuated in the presence of the GR–PR antagonist RU486.[160,203] GR upregulates $cPLA_2$ by direct binding to its promoter region in amnion fibroblasts, providing one mechanism for the enhanced PGE_2 synthesis.[204]

The rise in glucocorticoids that occurs in virtually all species late in gestation serves to enhance preparation of the fetus for survival by promoting organ maturation and stimulating PG production, which augments myometrial contractile activity and cervical ripening. This process may be prematurely activated with infection of the amnion and chorion, chorioamnionitis. Cytokines such as IL1β and tumor necrosis factor alpha (TNFα), released after pathogen detection through Toll-like receptors (TLRs) on macrophages, directly stimulate PG synthesis in the fetal membranes and also induce 11βHSD1 in the membranes by activating its transcription.[173,205,206] This induction of 11βHSD1 would serve to regenerate more cortisol, activate membrane PTGS2 and $cPLA_2$, and further enhance production of the myometrial contractile agonist PGE_2, accelerating labor.

Decidual activation and membrane rupture: In humans, the initiation of forceful, synchronous uterine contractions together with rupture of the fetal membranes comprise the critical mechanical components of parturition. These two aspects of delivery of the fetus appear to be controlled separately.[207] For the majority of women who give birth at term, uterine contractions are the initial manifestation, followed by spontaneous rupture of the fetal membranes.[208,209] This sequence is not invariable, as for 10% or more of women, fetal membrane rupture without contractions is the initial symptom of parturition. In the context of preterm birth, approximately one-third of women will experience preterm premature rupture of membranes (PPROM) without uterine contractions as their presenting complaint.[208,209] As the mechanisms initiating human parturition at term or preterm remain unclear, whether PPROM and spontaneous labor with contractions reflect distinct pathophysiologies or variations of the same underlying pathophysiology continues to be controversial.[210]

Through many recent investigations in animal model systems and humans, evidence for both biomechanical and biochemical influences that promote membrane rupture have emerged. Early studies found that membrane deformation and thinning due to uterine contractions during labor lead to their weakening.[211,212] These contractions result in membrane deformation such that the original starting condition is not obtained. In addition, prelabor, Braxton-Hicks contractions increase intrauterine pressure approximately 10 mm Hg and enhance biochemical-mediated weakening by both direct biophysical actions and the capacity of stretch

forces in the membranes to activate the transcription of remodeling genes, which will be discussed further in the "The Hormonal Regulation of Parturition" section.[213–215]

The biochemical mechanisms that contribute to membrane rupture include both matrix remodeling and cellular apoptosis.[149] The chief source of tensile strength in the amnion is collagen I, found in the compact layer and mesoderm, and collagen IV, found in the basement membrane and the connections between the amnion epithelium and the mesenchyme.[216,217] Degradation of collagen is critical to the progression of membrane rupture, and it is controlled by the expression of matrix MMPs and their inhibitors, TIMPs.[216] A number of MMPs have been found in the amnion and chorion, each with different specificity for collagen degradation; these include MMP1, MMP2, MMP3, MMP8, and MMP9.[218–220] MMP1 is the major isoform prior to the onset of labor[219] and may be induced by IL1β.[221] MMP2 is not altered in expression in association with impending membrane rupture at term or preterm.[218,220] MMP9, however, increases in amniotic fluid with PPROM, and is responsive to PGE_2, $PGF_{2\alpha}$, and TNFα.[222,223] MMP9 activity may serve as a reliable marker for impending membrane rupture.[224,225] Conversely, the inhibitor TIMP1, a key regulator of MMP9 activity, decreases with PPROM and labor.[218] As a consequence of the enhanced MMP activity, collagen fibers have been shown to be decreased at term, along with general disruption of their architecture.[224–226]

Extracellular matrix degradation due to MMP activity not only directly disrupts biomechanical integrity of the membranes but also leads to cellular apoptosis.[216] One mechanism involves the release of membrane-bound cytokines such as TNFα and Fas ligand, which further serves to enhance the production of MMPs and provides a positive-feedback loop for membrane rupture.[227,228] In humans, several polymorphisms in TNFα, IL1β, MMP1, MMP8, MMP9, and SerpinH1 (heat shock protein 47) have been associated with patients with PPROM that likely contribute to individual risk for preterm birth through accelerating this positive-feedback pathway.[229–234] In animal models, increased apoptosis of the amnion epithelium, along with other compartments, follows the induction of MMP transcription and activity.[235–237] In the rat, this matrix reorganization and apoptosis occur in a widespread fashion throughout the fetal membranes. In humans, this general pattern throughout the membranes has not been observed, but rather a focal area of morphological change, both during active labor and in women undergoing Cesarean section delivery without labor, in the fetal membrane region adjacent to the cervix.[238–240]

With the advent of technologies to carefully and systematically measure membrane biophysical characteristics, the sequence of events leading to rupture has become clearer.[149] In vivo, the lower pole of the fetal membranes separates from the decidua of the lower uterine segment and is associated with the release of fetal fibronectin, normally present at the site of chorionic-decidual contact.[224,239–241] Fetal fibronectin serves as a robust biomarker for both term and preterm parturition.[242–244] Controlled observation of membrane rupture ex vivo, after distention of membranes, revealed that the amnion initially separates from the choriodecidua.[241] The amnion and choriodecidua then rupture separately, with the choriodecidua, being the weaker component, rupturing first. The amnion then ruptures after further nonelastic deformation. Even prior to the onset of contractions, the weak zone overlying the cervix, exhibiting increased expression of MMP-9 and apoptosis, can be demonstrated and may serve as the target for membrane disruption as it has been found adjacent to the tear line after spontaneous membrane rupture.[239,245,246]

THE HORMONAL REGULATION OF PARTURITION

The process of parturition is initiated and controlled by a complex set of hormonal interactions that impact the myometrium, cervix, decidua, and fetal membranes to induce and coordinate the events described here. A common characteristic of the hormonal axes that affect parturition is the development of positive-feedback loops locally within the gestational tissues that produce tissue-level inflammation, and a neuroendocrine interaction that augments OT secretion by the maternal pituitary. A general principal in biological control systems is that self-amplifying loops usually lead to a terminal event that breaks the positive-feedback interaction and restores homeostasis. In this case, the terminal event is emptying of the uterus.

Function of the gravid uterus is controlled by the net effect of hormones that promote relaxation and quiescence and those that promote labor. For most of pregnancy, the balance is in favor of hormones promoting relaxation and quiescence. Parturition is triggered by a switch in the balance toward pro-labor hormones. In this context, the hormonal control of parturition is dominated by the steroid hormones progesterone and estrogen; the potent stimulatory uterotonins $PGF_{2\alpha}$, PGE_2, and OT; peptide hormones that are produced by the placenta and modulate responsiveness to uterotropins and uterotonins; and cytokines and chemokines produced as part of tissue-level inflammation in the myometrium, cervix, and decidua. In addition, biomechanical signals in response to uterine distention may contribute to pro-labor signals. Each of these factors contributes to the development of positive-feedback hormonal interactions within the gestational tissues, between the fetus and the mother, and between the uterus and the maternal pituitary that ultimately lead to labor and delivery.

Progesterone

In all viviparous species studied so far, progesterone is essential for the establishment and maintenance of pregnancy, and its withdrawal is a key trigger for parturition. The "progesterone block" hypothesis, proposed by Arpad Csapo in the 1950s,[2] posits that progesterone maintains pregnancy by blocking labor (i.e., it promotes myometrial relaxation, cervical closure, and decidual quiescence), and that parturition initiates when the block is withdrawn. Studies in multiple species, including humans, support this hypothesis and show that progesterone withdrawal is the central trigger event in the hormonal control of parturition.[247,248]

Progesterone needed to establish and maintain human pregnancy is initially provided by the maternal corpus luteum (CL), whose life span is prolonged by chorionic gonadotropin secreted by trophoblast cells of the blastocyst (luteolysis usually occurs at the end of the luteal phase of a nonfertile cycle). Progesterone also is produced by the developing villous syncytiotrophoblast, which by the 10th to 12th week of gestation increases in mass and produces enough progesterone to obviate the need for the CL. After this stage of pregnancy, referred to as the luteal–placental shift, the maternal ovaries can be removed without affecting the pregnancy. In contrast, removal of the ovaries prior to the luteal–placental shift causes miscarriage, which can be avoided by progesterone replacement therapy.[249,250] Production of progesterone by the villous syncytiotrophoblast of the placenta continues for the remainder of human pregnancy.

In most species (e.g., sheep, goat, rat, and mouse), parturition is triggered by a fall in maternal progesterone levels, referred to as systemic progesterone withdrawal, due to luteolysis in species that rely on the CL for progesterone, or modulation of placenta steroidogenesis in species in which the placenta is the source of progesterone. In humans and other species (including higher primates, guinea pig, and armadillo), placental progesterone production persists during parturition and decreases only when the placenta is delivered. In these species, the uterus transitions to the laboring state, even though it is exposed to high levels of progesterone.[251] Nonetheless, treatment with antiprogestins induces labor at all stages of pregnancy, consistent with the concept that withdrawal of the progesterone block triggers parturition.[252] To resolve this apparent paradox, it is proposed that parturition in humans and other species that lack a systemic progesterone withdrawal is triggered by a functional progesterone withdrawal whereby target cells in the myometrium, cervix, and decidua desensitize to the pro-gestational (i.e., labor-blocking) actions of progesterone. Thus, diversity in parturition control between species is due mainly to differences in (1) the source of progesterone (placenta or maternal CL) to maintain pregnancy, (2) the mechanism for progesterone withdrawal (systemic or functional), and (3) the upstream hormonal interactions that induce progesterone withdrawal.

The extent to which fetal and maternal hormones affect the parturition trigger mechanism differs markedly among viviparous species. In mice, luteolysis at parturition is induced by increased PG production by the maternal decidua. In this species, maternal signals, likely operated by a clock mechanism, determine the timing of birth. In contrast, parturition in sheep is triggered by the fetus (Figure 42.5). In this species, parturition is induced by a surge in cortisol production by the fetal hypothalamus–pituitary–adrenal (HPA) axis 1–2 weeks before term,[253] which alters placental steroidogenesis and leads to a dramatic decrease in placental progesterone secretion[254] and a concomitant increase in placental estrogen production by inducing activity of P450c17 (CYP17A1). The fall in circulating progesterone levels (i.e., systemic progesterone withdrawal), coupled with a rise in circulating estrogens, transforms the uterus and cervix to the laboring state. As cortisol is the principal stimulator of fetal organ system maturation, the prepartum fetal cortisol surge ensures that parturition is coordinated with fetal maturation. Although cortisol also stimulates the maturation of organ systems in the human fetus, it does not affect the parturition process. Administration of glucocorticoid to women in threatened preterm birth situations does not advance the parturition process, and parturition occurs normally at term in pregnancies where the fetus cannot produce cortisol.[255,256]

Progesterone promotes human pregnancy via its interaction with nuclear PRs in myometrial, cervical, and decidual cells. The importance of PR-mediated signaling for the maintenance of human pregnancy is reflected by the fact that administration of PR antagonists at any stage of pregnancy induces labor and rapid remodeling of the cervical ECM and loss of tensile strength.[257–261] This suggests that the progesterone block to parturition operates, at least in part, through progesterone- and PR-mediated mechanisms, and that inhibition or disruption of PR activity is sufficient to induce the full parturition cascade.

The human PRs exist as two major isoforms, PR-A (83 kD) and PR-B (99 kD). PR-A is a truncated (by 165 N-terminal amino acids) form of PR-B (Figure 42.6). Other PR transcripts generated by alternate translational start sites, exon splicing, intronic insertions, and exon deletions have also been described, but their levels of expression in most tissues, including the gestational tissues, are low, and their physiological significance is uncertain.[262]

PR-A and PR-B function primarily as ligand-activated nuclear transcription factors and affect target cell phenotypes primarily by modulating gene expression.[263] This genomic mode of action suggests that in

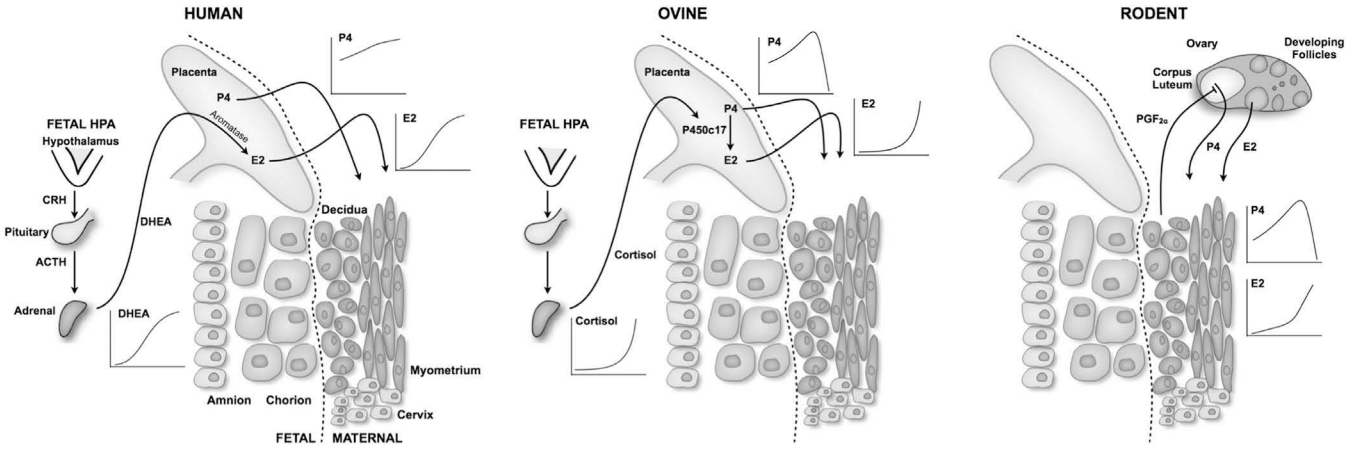

FIGURE 42.5 Comparison of tissue synthesis and changes in circulating progesterone (P4) and estradiol (E2) in human, ovine, and rodent pregnancy. In the human, the placenta produces progesterone throughout pregnancy, and there is no systemic decrease in P4 at parturition. In addition, the placenta produces estrogens (E2 (shown), estriol, estrone, and estetrol) from dehydroepiandrosterone (DHEA) supplied by the fetal adrenal cortex. In ovine pregnancy, the placenta is the principal source of P4. Late in gestation, activation of the fetal hypothalamic–pituitary–adrenal axis leads to a surge in cortisol that induces expression of P450c17 in the placenta, which converts P4 to androstenedione that can be used for estrogen synthesis. This causes a decrease in circulating P4 levels and a coordinated increase in circulating E2 levels. In rodent pregnancy, the maternal ovaries are the exclusive source of P4 (secreted by CLs) and E2 (secreted by developing follicles). At parturition, $PGF_{2\alpha}$ produced by the decidua induces luteolysis, leading to systemic P4 withdrawal, and E2 levels increase in response to further ovarian follicle development, which is in response to increased gonadotropin exposure due to removal of P4 negative feedback to the maternal hypothalamus and pituitary.

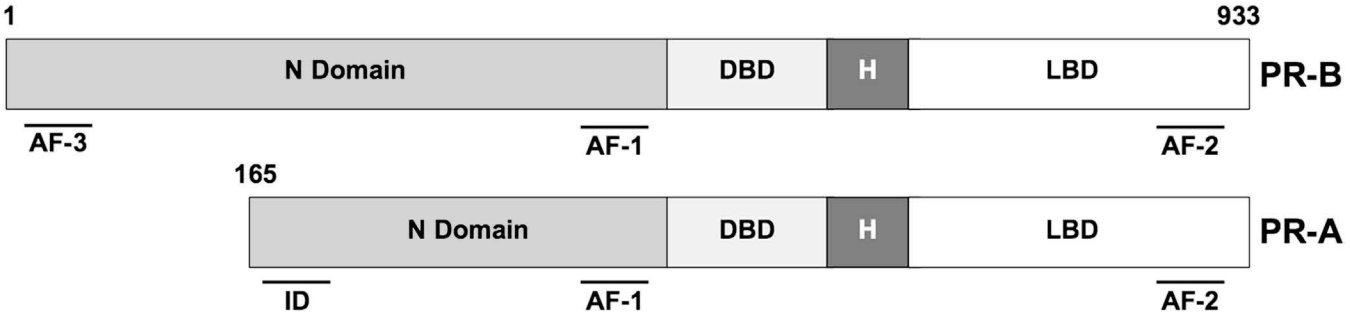

FIGURE 42.6 Organization of human PR-B and PR-A. The molecules can be divided into four functional domains: (1) the N domain at the N terminus, (2) the DNA-binding domain (DBD), (3) the hinge (H) domain, and (4) the ligand-binding domain (LBD). AF-1, AF-2, and AF-3 refer to transcription activation regions, and ID is the inhibitory domain that confers PR-A-mediated inhibition of PR-B.

myometrial, cervical, and decidual cells, the PRs promote the expression of genes encoding pro-gestational factors and inhibit the expression of pro-labor genes. However, the exact transcriptional mechanism is not clearly understood as the promoter regions of most parturition-related genes lack distinct progesterone response elements. In this case, progesterone may affect parturition-related gene expression indirectly via effects on other genes. It is also possible that the PRs modulate the abundance of specific micro-RNAs (miRNAs) that target pro-labor mRNAs and inhibit translation.[264–266]

Progesterone responsiveness in reproductive tissues involves the combined actions of PR-A and PR-B, each mediating distinct effects on specific gene targets (Figure 42.2). At some promoters, PR-A represses the activity of PR-B, and as such it is generally considered that the net transcriptional activity of PR-B is inversely related to the relative amount of PR-A and PR-B.[267,268] This concept led to the PR-A–PR-B hypothesis for functional progesterone withdrawal,[269] which posits that pro-gestational actions of progesterone are mediated by PR-B and that functional progesterone withdrawal at parturition is caused by an increase in the myometrial cell PR-A:PR-B ratio due to increased expression of PR-A.[269] Consistent with this hypothesis, PR-A represses the transcriptional activity of PR-B in human myometrial cells,[269–273] and the onset of labor is associated with increased PR-A expression in myometrial cells leading to a rise in the PR-A:PR-B ratio.[269,270,273,274]

Functional progesterone withdrawal also may be mediated by other mechanisms that decrease PR-induced

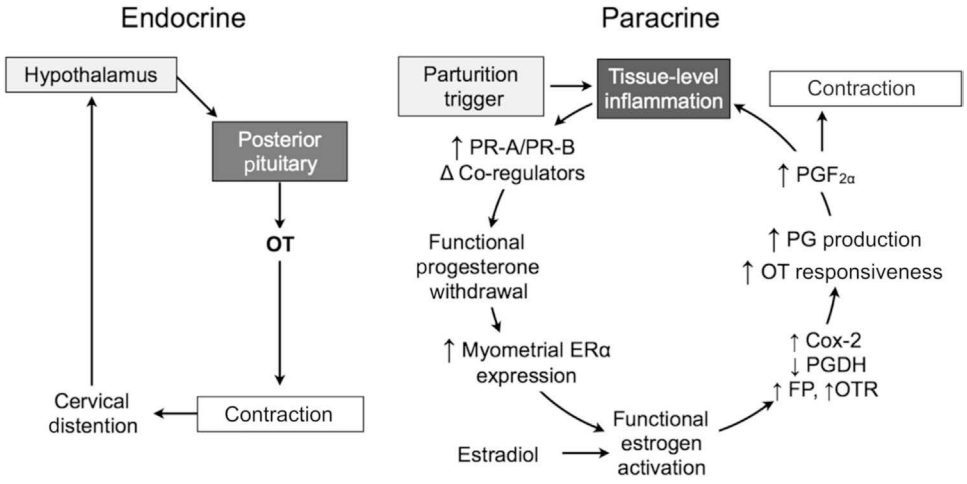

FIGURE 42.7 Positive-feedback hormonal loops in the physiology of parturition. Left: Endocrine feedback loop involving OT secreted from the hypothalamus–posterior pituitary in response to cervical distention induces contractions that further distend the cervix to increase pituitary OT release. Right: Paracrine feedback loop within the gestational tissues. Tissue-level inflammation induces functional progesterone withdrawal, which induces functional estrogen activation, which increases responsiveness to estrogens that augment PG synthesis and responsiveness to OT. PGs induce contractions and cervical ripening and augment tissue-level inflammation.

transcriptional activity. For example, critical PR co-activators are decreased in the myometrium in association with the onset of labor, and as such the capacity for ligand-activated PR-B to control gene expression could be compromised.[275] The transcriptional activity of PR-B also may be blocked by the NFκB transcription factor complex.[276,277] This mechanism may be active in infection-associated preterm labor since NFκB is a major mediator of the inflammatory response.[278–281] Progesterone withdrawal also may be mediated by the conversion of progesterone to a less active form with diminished progestin activity. Studies in transgenic mice and human tissue show that at the time of labor, conversion of progesterone to the inactive 20α dihydroprogesterone increases in endocervical fibroblasts.[282,283] Thus, current data indicate that in human parturition, the progesterone block to the onset of labor is removed by a combination of biochemical events that abrogate the capacity for PR-B to mediate pro-gestational actions of progesterone.

Multiple studies have shown that parturition is an inflammatory process.[284,285] During the prelude to labor, the myometrium, cervix, and decidua exhibit edema, neutrophil infiltration, and expression of chemical mediators of inflammation, especially proinflammatory cytokines, chemokines, and PGs.[133,134,286] It is now generally accepted that inflammatory mediators and intrauterine inflammation are key stimulators of parturition. In fact, stimuli such as mechanical distention and infection, which commonly induce proinflammatory cytokine expression and inflammation, are associated with premature delivery and are often effective as abortifacients. Implicit in the hypothesis that labor is induced by intrauterine inflammation is the concept that uterine quiescence ensues if proinflammatory pathways in myometrial, cervical, and decidual cells are repressed. In the 1970s, Siiteri and colleagues proposed that progesterone promotes pregnancy maintenance by suppressing proinflammatory responses in the gestational tissues.[287] Recent studies have shown that progesterone indeed represses inflammation in the gestational tissues via its interaction with specific PR isoforms[271,277] and with the glucocorticoid receptor.[288] Importantly, antiinflammatory actions of progesterone in myometrial cells were mediated by PR-B, and this effect was inhibited by PR-A (Figure 42.2).[271] Based on those observations, it is proposed that withdrawal of PR-B-mediated transcriptional and antiinflammatory activity by increased expression of PR-A allows for the activation of proinflammatory pathways, which induces tissue-level inflammation that transforms the myometrium, cervix, and decidua to the laboring state (Figure 42.7).

Understanding the hormonal control of parturition requires elucidation of the hormonal interactions that induce progesterone withdrawal. In sheep (Figure 42.5), this pathway is well characterized.[253,289,290] In human parturition, the pathway is less clear. Based on the PR-A–PR-B hypothesis for functional progesterone withdrawal, it is possible that parturition is induced by factors that augment PR-A expression and/or activity in the gestational tissues. Studies examining the control of PR-A expression in human myometrial cells suggest that it is upregulated by $PGF_{2\alpha}$,[291] a key uterotonic hormone whose production by the gestational tissues increases before labor.[292,293] Clinical studies show that administration of $PGF_{2\alpha}$ induces labor at all stages of human pregnancy.[294,295] Thus, it is possible that $PGF_{2\alpha}$ induces labor by first increasing myometrial cell PR-A expression to cause functional progesterone withdrawal. This paradigm suggests that any process that increases exposure of the gestational tissues to $PGF_{2\alpha}$ (e.g., uterine distention, intrauterine infection, or fetal membrane PG production) could induce functional progesterone withdrawal. A threshold level of PR-A likely exists, above which it represses the antiinflammatory activity of PR-B. At this point, a positive-feedback proinflammatory loop develops within the gestational tissues that further increases the inflammatory state, leading to

a further increase in $PGF_{2\alpha}$. Eventually, the $PGF_{2\alpha}$ levels increase to a point whereby the hormone exerts potent uterotonic actions to induce labor. This positive-feedback proinflammatory interaction may explain why labor is difficult to stop once it initiates. Further studies are needed to fully elucidate the molecular links between PR-mediated progesterone signaling and inflammation in the physiology of human parturition.

Evidence from in vivo and in vitro studies supports the hypothesis that progesterone nongenomically influences myometrial contractility via its interaction with specific membrane PRs (mPRs) (Figure 42.2) linked with intracellular signaling cascades.[296] In contrast to nuclear PRs that affect target cell function by modulating gene expression, the mPRs mediate a rapid direct response to progesterone. In one of the first clinical trials of progestin tocolysis, Hendricks et al.[297] found that administration of a large bolus of progesterone into the amniotic fluid of women in active labor at term almost immediately decreased contraction frequency and responsiveness to OT. In the mid-1960s, Pinto et al.[298] reported that large amounts of progesterone (100–200 mg bolus IV; this is close to the amount that the placenta produces in a day) administered to women in active labor at term inhibited the frequency and intensity of uterine contractions within minutes. Importantly, Pinto and colleagues later found that progesterone exerted the same relaxatory effects on isolated myometrial strips.[299] However, subsequent studies on myometrial strips produced conflicting outcomes, with some reporting a rapid relaxatory effect of progesterone,[299–303] while others reported augmented contraction frequency but decreased contraction duration and amplitude.[304–306] The reason for this variability could be due to differences in the contractile state of the tissue at the time of collection (i.e., relaxed or in active labor), and differences in the methods used to prepare the progestin solutions and perform the contraction measurements. Nonetheless, the studies clearly demonstrated that progesterone exerts rapid effects on myometrial contractility, typical of a direct nongenomic mode of action.

In recent years, specific mPRs have been identified. The mPRα, mPRβ, and mPRγ family are derived from separate genes, and are structurally related to seven-transmembrane G-protein coupled receptors.[307,308] mPRα is expressed in the myometrium from the lower uterine section, its expression increases with the onset of active labor,[309] and studies in primary cultures of term myometrial cells showed that mPRα and mPRβ are coupled with inhibitory G-proteins and increase contractility by decreasing cAMP and augmenting the transcriptional activity of PR-B.[310] However, controversy exists regarding the roles of mPRα, mPRβ, and mPRγ in the human pregnancy myometrium because some studies in a variety of cell types have found that these mPRs do not localize to the plasma membrane and are not activated by progesterone.[311]

Another family of mPRs are progesterone receptor membrane component 1 and 2 (PGRMC1 and PGRMC2), which have a single transmembrane domain and a steroid-binding extracellular domain.[312–314] PGRMC1 is expressed in the lower uterine section of the human myometrium and may mediate the relaxatory actions of progesterone.[315] Levels of PGRMC1 decrease in the myometrium in association with the onset of labor, and inhibition of PGRMC1 signaling in term myometrial strips suppresses progesterone-induced relaxation.[315] In granulosa cells, binding of progesterone to PGRMC1 activates PKG and decreases intracellular free Ca^{2+} levels.[316] Similar outcomes were observed in sensory neurons in which PGRMC1 appeared to mediate progesterone-induced inhibition of Ca^{2+} accumulation.[317] Interestingly, studies in primary cultures of term myometrial cells showed that progesterone rapidly decreases intracellular free Ca^{2+} levels[300,318] and increases cAMP,[319,320] nongenomic actions that would be expected to promote relaxation.

In addition to the direct regulation of gene transcription, ligand-activated steroid hormone receptors can also activate cytosolic signaling cascades. This "extranuclear" activity of steroids is characterized by rapid activation (within seconds to minutes) that does not require de novo gene transcription and occurs without the translocation of the steroid receptor to the nucleus. Nongenomic actions of progesterone may be mediated by extranuclear actions of PR-A and PR-B. Studies to determine the mechanism by which progesterone exerts tropic actions in breast cancer cells revealed that ligand activation of PR-B rapidly activated the extracellular signal-regulated kinase (ERK)-signaling pathway (the MAPK pathway) by interacting directly with c-Src.[321,322] c-Src is a key intermediate that couples hormone (mainly growth factor) signaling through plasma membrane receptors with intracellular transduction pathways involved in regulating cellular processes such as proliferation, differentiation, adhesion, migration, and apoptosis.[321] Studies in breast cancer cells show that only PR-B activates c-Src; PR-A has no effect, and PR-B interacts directly with ERα to form a complex that also initiates the Src–Ras–ERK cascade.[323] Thus, it is possible that progesterone and estrogen promote uterine growth via a mutual extranuclear interaction that activates the Src–Ras–ERK cascade. In contrast, effects on contractile phenotype may be limited to genomic pathways and mPR-mediated nongenomic mechanisms.

Estrogens

Estrogens exert uterotropic effects on the gravid uterus. They promote protein synthesis, tissue growth, and blood flow in the myometrium, decidua, and cervix,

and, in conjunction with progesterone, modulate maternal physiology to favor the pregnant state.[324–328] Thus, for most of pregnancy, estrogens function as pro-gestational agents. This activity ceases, however, at parturition when estrogens oppose the pro-gestational actions of progesterone by promoting the expression of genes in the myometrium, decidua, and cervix whose products increase contractility and promote cervical ripening and membrane rupture.[324] At parturition, estrogens increase the responsiveness of myometrial cells to uterotonic agents such as OT and PGs,[329–331] increase the production and release of PGs by the gestational tissues and fetal membranes,[332] and increase the formation of gap junctions between myometrial cells[333–337] (Figure 42.2). Estrogens also promote cervical ripening and dilation and membrane rupture by stimulating the expression of proteolytic enzymes (e.g., collagenase) by endocervical fibroblasts and decidual cells, respectively.[121,122]

In most species, maternal estrogen levels are low during most of pregnancy and rise prior to, and in some cases in conjunction with, progesterone withdrawal at parturition.[338] In humans and higher primates, however, levels of estrogens (estrone, estradiol, estriol, and 15α-hydroxyestriol (estetrol)) are high for most of pregnancy and increase further over the final weeks of gestation.[251,338] The principal source of estrogens in human pregnancy is the placenta, which expresses high levels of the aromatase enzyme and, as such, efficiently converts C19 androgens (mainly dehydroepiandrosterone sulfate (DHEA-S)) to estrogens.[339–341] The human placenta, however, cannot synthesize C19 steroids and therefore is dependent on other steroidogenic organs, primarily the fetal adrenal, to supply precursor androgens for estrogen synthesis. This steroidogenic axis is referred to as the feto-placental unit. Estrone and estradiol are produced from DHEA-S, supplied in roughly equally amounts by the maternal and fetal adrenals,[342] and estriol (produced from 16-hydroxy-DHEA-S) and estetrol (produced from 15-hydroxy-DHEA-S) are produced from fetal adrenal DHEA-S that is hydroxylated at the 16 and 15 positions, respectively, by the fetal liver.[340,343] Thus, maternal levels of estriol and estetrol—and, to a lesser extent, estradiol and estrone—reflect activity of the fetal HPA axis.

Congenital abnormalities affecting the feto-placental unit provide insight into the role of placental estrogens in the regulation of human parturition. Increased placental estrogen synthesis occurs in pregnancies in which the fetal adrenals cannot synthesize cortisol due to 21-hydroxylase deficiency. In these pregnancies, fetal adrenocorticotropic hormone (ACTH) levels are elevated due to a lack of cortisol negative feedback on the fetal hypothalamus and pituitary, leading to increased production of DHEA-S that is converted by the placenta to estrogens. Despite the elevated estrogens throughout most of pregnancy, these pregnancies deliver normally at term.[255] Thus, increased estrogen synthesis by the feto-placental unit beyond the already high level does not induce parturition. Likewise, the timing of parturition is not markedly affected by abnormalities that decrease placental estrogen production (i.e., anencephaly,[344,345] congenital adrenal lipoid hyperplasia,[346,347] placental aromatase deficiency,[348–352] and placenta sulfatase deficiency[353–355]). Those experiments of nature, however, do not exclude a role for estrogens in the parturition process, as in most cases of decreased placental estrogen synthesis, the levels of maternal estrogens, although low compared with those of normal pregnancies, are still in a physiologically significant range (1–1.6 nmol/l) and comparable to levels reached in the midcycle and luteal phases (0.6–2.0 nmol/l). In this regard, it is noteworthy that no natural conditions have been identified in which parturition occurs in the complete absence of estrogens. Thus, under normal conditions, estrogen levels in human pregnancy far exceed the amounts needed to effect parturition. In this highly estrogenic milieu, the uterine tissues are refractory to estrogen-induced pro-labor gene expression for most of pregnancy. A key question therefore is: how are pro-labor estrogenic actions on the gestational tissues activated at parturition? A likely scenario is that in human pregnancy, estrogen action is regulated by target tissue responsiveness rather than absolute circulating levels. If this is the case, then the source of estrogen may be of lesser importance provided that estrogenic drive is appropriate when target cell responsiveness to the pro-labor mode of estrogen action is increased (i.e., functional estrogen activation).

The regulation of estrogen responsiveness in the gestational tissue likely involves modulation of ER abundance or transcriptional activity and may be affected by progesterone. In most estrogen- and progesterone-responsive tissues, progesterone decreases estrogen responsiveness by inhibiting expression of ERα.[269,356–360] In the rhesus monkey, treatment with RU486 at midgestation increases myometrial expression of ERα,[357] and in human pregnancy myometrial ERα expression increases during the prepartum period and in association with PR-A-mediated functional progesterone withdrawal.[269] These data suggest that one mechanism by which progesterone maintains pregnancy is by blocking estrogen-induced expression of pro-labor genes through inhibition of ERα expression in the gestational tissues. According to this paradigm, progesterone withdrawal at parturition leads to an increase in ERα expression, which increases the responsiveness of the gestational tissues to pro-labor actions of circulating estrogens. This reciprocal interaction would effectively synchronize progesterone withdrawal and estrogen activation (i.e., functional progesterone withdrawal induces functional estrogen

activation), provided that the myometrium is exposed to estrogenic hormones.

This model does not explain how estrogens exert uterotonic actions on the gravid uterus, especially as ERα is barely detectable in the human pregnancy myometrium.[87] One possible explanation for this conundrum is that estrogens interact with splice variants of ERα that signal via extranuclear nongenomic mechanisms.[361] Estrogens have been shown to directly regulate the activity of multiple intracellular signaling pathways, including the ERK pathway, the phosphatidylinositol 3-kinase (PI3K)–protein kinase B (PKB) pathways, and Gα subunit signaling.[321] Activation of the ERK signaling pathway by estrogens has been extensively studied in breast cancer cells, where this cascade is thought to mediate the proliferative response to estrogens.[362] Further studies are needed to determine the role of this signaling pathway and the specific ERs involved in mediating estrogenic actions on the gestational tissues of the gravid uterus in the context of pregnancy maintenance and parturition.

Oxytocin

OT is a nine-amino-acid peptide with potent uterotonic activity; it markedly increases the frequency and force of myometrial contractions. OT is primarily a neuropeptide, synthesized by magnocellular neurosecretory cells in the supraoptic and paraventricular nuclei of the hypothalamus and stored in axon terminals that project to the posterior pituitary. In the gravid uterus, OT is also produced by the decidua and, to a lesser extent, the amnion and chorion.

During pregnancy, OT is inactivated by a cystine aminopeptidase, known as oxytocinase, produced by the decidua, chorion, and placenta.[363–368] The extent to which OT affects the myometrium is therefore dependent on the balance between peptide production in the brain and decidua and catabolism by oxytocinase. The rapid and efficient breakdown of OT is thought to limit the amount of intact OT that accesses the myometrium. Combined with the pulsatile release of OT from the maternal posterior pituitary during labor,[369] this metabolic barrier may ensure intermittent stimulation of the myometrium, especially during the phasic contractions of labor.

OT is secreted from the posterior pituitary in response to cervical and vaginal distention during labor.[370,371] It is also secreted as part of the milk letdown reflex during lactation. During labor, OT secreted by the posterior pituitary is part of a positive-feedback neuroendocrine loop whereby distention of the cervix due to the contractions of labor induces the release of OT, which stimulates the myometrium to contract to further distend the cervix (Figure 42.7). Secretion of OT by the maternal posterior pituitary also represents a mechanism for maternal control of certain aspects of parturition, including circadian timing and response to environmental stressors.[372]

The role of intrauterine OT is less clear. OT released from the decidua may gain access to adjacent targets, including the myometrium, via a paracrine route.[373,374] Intrauterine OT production, however, is low compared with the amount released by the posterior pituitary during active labor and in response to cervical distention.[375] Moreover, the amount of local OT that reaches the myometrium is uncertain as the decidua and chorion express high levels of oxytocinase. Nonetheless, it is clear from studies of animals hypophysectomized during pregnancy that OT from other sources contributes to the parturition cascade. OT production by the decidua is upregulated by estrogen.[376] This suggests that local OT contributes to estrogen-induced transformation and activation of the uterus. Interestingly, the OTR also is expressed in the human decidua,[367] and OT stimulates the production of PGs by decidual cells.[377] Taken together, the data suggest that OT produced by decidual cells in response to estrogens acts in an autocrine manner to increase the production of PGs that could in turn affect the myometrium. Thus, locally produced OT may serve to mediate pro-labor actions of estrogens during the prelude to active labor and amplify a positive-feedback loop within the gestational tissues involving PG production by the decidua.

The direct uterotonic actions of OT on the myometrium occur predominantly during the expulsive phase of active labor. In this regard, the positive-feedback loop between OT release from the posterior pituitary and cervical–vaginal distention plays a central role during labor. OT is commonly administered to women in whom augmentation of labor is needed. The potency of OT is reflected in the increased risk of abruption and hemorrhagic detachment of the placenta from the uterine wall, if excessive amounts of OT are administered.[378] In contrast to its effects during active labor, OT treatment is far less effective at inducing labor. For induction of labor, OT is usually used as a supplement to procedures such as amniotomy that trigger the parturition cascade by provoking tissue-level inflammation.

Although maternal pituitary OT is a potent stimulatory uterotonin during active labor, it is not essential for the successful completion of labor and delivery. Birth occurs normally in women with posterior pituitary dysfunction,[379] reflecting redundancy in the parturition process. In animal models, pharmacologic inhibition of OT delays but does not abolish parturition,[380,381] and transgenic mice lacking OT have a normal parturition,[382,383] although pups from OT-null mice die because the mother cannot produce milk.[383] Thus, OT is essential for lactation, but redundant mechanisms, possibly mediated by

vasopressin, operate to allow successful parturition in its complete absence.

Actions of OT in the uterus are mediated by the OTR, a Gq-coupled receptor, expressed by myometrial and decidual cells mainly at the end of pregnancy.[384] In myometrial cells, activation of the OTR leads to an increase in intracellular Ca^{2+} that stimulates contractions (as discussed in this chapter). In decidual cells, activation of the OTR by OT leads to increased expression of PTGS2 and production of PG.[377] OTR expression in the gravid uterus, and especially in the myometrium, is increased at term and in response to estrogens and progesterone withdrawal.[370,385–387] Uterine stretch also increases OTR expression in myometrial cells.[388]

Before active labor, levels of OT in the maternal circulation do not increase substantially,[389,390] and the extent of OTR expression in the myometrium and decidua is relatively low.[391] As part of the parturition cascade, OTR expression in the myometrium and decidua increases in response to progesterone withdrawal, increased estrogenic stimulation, and stretch, and as a consequence, the responsiveness of tissues to OT increases.[392] In rats, OTR is induced by locally produced PGs,[393] and activation of the OTR in endometrial cells increased PG production.[394] These data suggest a complex positive-feedback loop between estrogens, locally produced PGs, the uterine OT system, and OT responsiveness in the gestational tissues. Animal studies show that disruption of OTR signaling represses labor and delays parturition,[370] and clinical trials of OTR antagonists show that inhibition of OTR can be used to repress preterm labor.[395–400]

Thus, the role of OT in parturition is complex. It is a potent uterotonin that contributes to the contractions of labor during the expulsive phase. It is part of a maternal positive-feedback neuroendocrine loop that augments labor and possibly synchronizes labor with circadian rhythms. It is also produced within the gravid uterus and appears to be involved in the regulation of PG production within the gestational tissue. One of the major functions of estrogens in preparing the uterus for labor is the stimulation of myometrial and decidual OTR expression and the stimulation of OT production by decidual cells. Despite its key actions during labor, OT is not essential for the successful completion of labor. This likely reflects redundancy in the parturition control process.

Prostaglandins

PGs exert important labor-promoting actions in all mammals.[165,166,401] In addition to augmenting uterine contractions, PGE_2 and $PGF_{2\alpha}$ may serve as components of the timing mechanism that initiates labor.[166,402,403] In humans and rodents, amniotic fluid and uterine tissue PGE_2 and PGF_{2a} concentrations rise shortly before the onset of labor.[167,404–407] Moreover, PG synthesis inhibitors or receptor blockers delay the timing for onset of parturition in both human and animal studies, including spontaneous preterm labor in humans.[166,167,408,409] Understanding the biology for regulation of PG production and degradation provides an important avenue for elucidating the clock that meters the timing for birth and identifying potential therapeutic strategies to prevent preterm birth.

As summarized in the "The Decidua and Fetal Membranes" section of this chapter, the synthesis of PGs is a complex, multistep process that is tightly regulated to compartmentalize their actions both spatially and temporally. The initial release of the requisite substrate for PG biosynthesis, arachidonic acid, from membrane-anchored phosphatidylethanolamine and phosphatidylinositol occurs through the action of either phospholipase A_2 or phospholipase C[410,411] (Figure 42.4). Of these two phospholipases, PLA_2 exerts the more robust role in parturition. The PLA_2 enzymes constitute an approximately 30-member family of related enzymes that display different cellular and subcellular localizations and actions.[410,411] The chief groupings among these isoforms are into cytosolic calcium-dependent (c)PLA_2s, calcium-independent (i)PLA_2s, platelet-activating factor acetylhydrolases, lysosomal PLA_2s, adipose-specific PLA_2s, and secretory (s)PLA_2s. Of the PLA_2s, group IV cPLA_2 appears to be the primary mediator of parturition-associated PGs, as is it selective for phospholipids containing arachidonic acid at the sn-2 position of membrane glycerophospholipids,[155,161,412] although some sPLA2 isoforms are also present in amnion and other gestationally relevant tissues.[155] While it has been found that sPLA_2 is secreted from fetal membranes, the myometrium, and the placenta, the contributions of sPLA_2 to the progression of labor remain unclear. Type II sPLA_2 is the primary isoform, and it has been found to increase with labor in some studies in the placenta, amnion, and choriodecidua.[413,414] Once released from membrane phospholipids, arachidonic acid can be metabolized through either the PG synthetic pathway (by the action of PTGS1 or PTGS2) or the lipoxygenase pathway to generate leukotrienes and related compounds.[9,162,415] The PG pathway is critical for the onset and progression of labor. PTGS1 and PTGS2 convert arachidonic acid to PGH_2, the first committed precursor for bioactive prostaglandin synthesis. These enzymes are highly homologous, sharing 60–65% amino acid identity within a species and similar capacity to convert arachidonic acid to PGH_2.[415,416] A splice variant in human PTGS1, designated cyclooxygenase-3, has also been described.[417] Cyclooxygenase-3 may play an important role in fever generation and attenuation by acetaminophen and related compounds,[417] but its specific role in the genesis of parturition-associated PGs has not been evaluated. PTGS1 and PTGS2 demonstrate significant differences in their patterns of expression. PTGS1 is expressed

by most tissues, and in most circumstances it exhibits little change in level of expression; hence, it has largely been considered a "housekeeping" gene. In contrast, PTGS2 is often undetectable at baseline but is highly induced by inflammatory signals, such as TLR engagement, cytokines, growth factors, and tumor promoters.[415]

In the mouse, PTGS1 in the uterine epithelium is the primary source of term parturition-promoting $PGF_{2\alpha}$ during late gestation.[405] In contrast to its typical "housekeeping gene" regulatory profile, PTGS1 mRNA is induced 40-fold during gestation, and 10-fold precisely between days 14.5 and 15.5 of mouse gestation (term at day 19.5).[418] In contrast, with infection- or inflammation-induced preterm labor in mice, uterine PTGS2 generates $PGF_{2\alpha}$, which mediates luteolysis and uterine contractions.[419] During human gestation, PTGS1 expression does not vary considerably in the uterus, placenta, and fetal membranes.[167,420–422] PTGS2 expression is highly induced in the amnion and appears to be the driver for labor-promoting PGE_2 during human term and preterm labor.[167,420–422] PGH_2 undergoes conversion to PGI_2, PGD_2, thromboxane A_2, PGE_2, and $PGF_{2\alpha}$ by specific isomerases that constitute several multiisoform families of diverse structure and regulation.[423–426] PGI_2, prostacyclin, is a major product of cyclooxygenase activity in the myometrium during pregnancy, and serves to relax uterine smooth muscle.[427,428] Changes in PGI_2 with parturition have not been delineated, although PGI_2 synthase decreases with advancing gestational age.[429] The enzymes generating PGE_2 and $PGF_{2\alpha}$ have garnered the greatest attention for parturition effects.[401] Prostaglandin E synthases (PGES) comprise a group of at least three different enzymes. Two of these isoforms are membrane bound (mPGES1 and mPGES2). mPGES1 can be induced by proinflammatory stimuli and has been shown to be functionally coupled with PTGS2.[430] mPGES2 is initially synthesized as a Golgi-associated protein and is further processed to a mature cytosolic enzyme after the cleavage from its N-terminal hydrophobic domain.[431] mPGES2 is functionally coupled with both PTGS1 and PTGS2. Cytosolic PGES (cPGES) is constitutively expressed in a wide variety of cells and is functionally linked to PTGS1.[430] $PGF_{2\alpha}$ can be synthesized from three different pathways starting at PGE_2, PGD_2, or PGH_2. The PGF synthases utilizing PGH2 as substrate are part of the aldo-ketoreductase superfamily and undergo upregulation during late gestation in the mouse.[418,424]

PG receptors have contrasting roles on the myometrium[165] and, in those species undergoing luteolysis, on the CL of the ovary.[432] Each PG acts on a specific group of receptors—PGE_2 on $EP_{1–4}$ (prostaglandin E receptors 1 through 4), $PGF_{2\alpha}$ on FP, prostacyclin on IP, thromboxanes on the thromboxane receptor (TP), and PGD on the prostaglandin D receptor (DP) (Figure 42.4). For myometrial function, PG receptors can either relax (IP, DP, EP_2, and EP_4 receptors) or contract (EP_1, EP_3, and FP receptors) uterine smooth muscle.[165,166] Similarly, on the CL, PGE_2 promotes luteal function through actions on EP_2 and perhaps other receptors,[432,433] while $PGF_{2\alpha}$ causes luteolysis through FP.[403] All PG receptors are G-protein coupled receptors that stimulate the production of IP3 and intracellular Ca^{2+} release when signaling through Gq (e.g., FP and EP_1), or they stimulate (e.g., IP, DP, EP_2, and EP_4) or inhibit production (e.g., EP_3) of cAMP when signaling through $G\alpha_s$ or Ga_i, respectively.[434,435] The consequences of exposure to PGE_2 or $PGF_{2\alpha}$ for myometrial or CL function depend upon timing during gestation, as receptor subtypes differ in their patterns of temporal expression. Studies applying PG receptor agonists and antagonists to human term myometrium demonstrated the presence of the contractile receptor subtypes EP_3, FP, and TP and relaxation-promoting subtypes EP_2, DP, and IP.[436,437] More detailed temporal expression of FP and EP2 receptor mRNAs has been performed in both rat and human myometrium.[438,439] In rat myometrium, EP_2 was highest in expression at day 16 of gestation and declined as delivery approached at day 22.[439] In contrast, FP mRNA expression was low at day 16 and increased significantly until the day of delivery, consistent with its inclusion as a CAP.[439] The induction of FP is inhibited by progesterone but not altered by uterine stretch.[440] Immunohistochemical localization of FP has demonstrated a redistribution of FP protein from both nuclear and cytosolic to primarily cytosolic as term approaches.[441] Redistribution of EP_2 in the rat myometrium has also been observed, with notable cell membrane localization near term.[165] In human myometrium, EP_3 and FP receptors decrease during Phase 0 of pregnancy compared to the nongravid state, consistent with a period of uterine quiescence.[442] From mid- to late gestation, EP_2 expression decreased, as did FP in the lower uterine segment of the human myometrium.[438] The decline in FP in the lower uterine segment is consistent with the need for relaxation of this component of the uterus, in contrast to the needed increase in contractile activity of the fundus to expel the fetus. Indeed, PGE_2 and $PGF_{2\alpha}$ stimulate contractions on the myometrium derived from the upper uterine segment, while PGE_2 inhibited and $PGF_{2\alpha}$ had no effect on contractility in the human myometrial lower segment.[443,444] These findings, together with an increase in EP_4 in the lower segment,[165] suggest a regionalization of PG receptor expression in the human myometrium to enhance net contractile direction toward emptying the uterus toward the cervix. Regionalization of PG receptors has also been found in the baboon uterus.[445,446] While regulation of specific isoforms differed, the net consequence was enhanced contractile function in the fundus as compared to the lower uterine segment.

To limit the action of PGs during pregnancy, they undergo metabolism by the nicotinamide adenine dinucleotide-dependent enzyme HPGD.[175,447] The activity of this enzyme produces 15-keto and 13,14-dihydro-15-keto molecules that have substantially diminished signaling capacity through the PG receptors. The abundant expression of HPGD in the chorion may act to specifically restrict the amnion effects of PGE_2, such that the myometrium and decidua are not activated.[157,448] Analysis of the regulation of HPGD in the chorion during human gestation reveals decreased expression during active term labor compared to women at term but not in labor, and decreased expression in spontaneous idiopathic or infection-associated preterm labor compared to gestation-matched controls.[157,158] Expression in the baboon is quite similar to that in humans, with HPGD found in the chorion, decidua, uterine lower segment, fundus, and cervix.[449,450] HPGD mRNA expression decreases in the decidua, fundus, and cervix through late pregnancy and labor.

Cytokines and Chemokines

Multiple studies have shown that human parturition is an inflammatory process characterized by increased proinflammatory cytokine production[133,286]; increased infiltration of neutrophils and macrophages into the myometrium, cervix, and decidua[134,135]; and activation of the NFκB[279] within the myometrium, decidua, and cervix.[284,451] Proinflammatory cytokines and chemokines, especially IL1, IL6, IL8, and tumor necrosis factor alpha (TNFα), appear to play key roles in the parturition process, although they do not exert uterotonic effects directly.[452,453]

The most likely mode of action for local cytokines in the parturition cascade is that they promote tissue-level inflammation and local production of PGs that then induce myometrial contraction, cervical ripening, and membrane rupture.[454,455] In the rhesus monkey, activation of the inflammatory system in the gestational tissues, including the myometrium, precedes the onset of labor,[456,457] and studies in rhesus monkeys and mice have shown that administration of pro-inflammatory cytokines or factors that induce a proinflammatory response initiates preterm labor.[455,458–460] Moreover, stimuli such as mechanical or chemical trauma and infection that commonly induces the expression of proinflammatory cytokines and chemokines leading to tissue-level inflammation, are associated with premature delivery and are often effective as abortifacients. A significant proportion of preterm births are associated with intrauterine infection,[461,462] clinically silent upper genital tract infection, and bacterial vaginosis.[285,463,464]

A key player that may be pivotal in this process is the NFκB transcription factor complex, which, in response to inflammatory cytokines, increases the expression of key CAP genes, including PTGS2, GJA1, FP, and OTR in myometrial cells.[279] As described here, progesterone may prevent labor indirectly by inhibiting tissue-level inflammation through repression of NFκB activity. According to this model, progesterone withdrawal releases inhibition of NFκB, making it susceptible to activation by local proinflammatory cytokines.

Studies in human myometrial cell lines show that $PGF_{2\alpha}$ stimulates PR-A expression and increases the PR-A:PR-B expression ratio.[291] The functional link between tissue-level inflammation and PR-A-induced functional progesterone withdrawal may be a mechanism through which inflammation in response to danger signals (e.g., intrauterine infection, fetal stress or maturation, and uterine wall overdistention) induces labor (Figure 42.7).

Placental Peptide Hormones

The general structure and function of the placenta are the subjects of Chapters 39 and 40. As detailed therein, the placenta produces a plethora of protein (including cytokines and growth factors) and steroid hormones, which it secretes, usually in large quantities, into the maternal circulation.[465] In general, most hormones produced by the placenta are pro-gestational and serve to adjust maternal physiology, usually in favor of pregnancy maintenance (e.g., progesterone) and to support the resource needs of the fetus (e.g., placental lactogen). With regard to the control of parturition, certain placental hormones may play a critical role. One of those is CRH.

CRH is a 41-amino-acid peptide that stimulates the expression and processing of proopiomelanocortin (POMC) by pituitary corticotropes and the secretion of ACTH. The human placenta, fetal membranes, and decidua also express CRH that is identical to that produced by the hypothalamus.[466] CRH expression by syncytiotrophoblasts can be detected from the seventh week of pregnancy and increases progressively until term.[467] Several actions have been ascribed to placental CRH in the control of human pregnancy.[468] CRH may serve an autocrine–paracrine function within the placenta by regulating expression and processing of POMC.[469] In this context, placental CRH may be part of the fetal–placental stress response. Neurotransmitters and neuropeptides activated in response to stress stimulate placental CRH release in vitro.[470,471] The physiologic implication of this is that the fetus may be able to mount a stress response via placental CRH. This may be critical in conditions such as preeclampsia, placental vascular insufficiency, and intrauterine infection.

Placental CRH is released mainly into the maternal compartment. Levels of CRH in the maternal circulation

can be detected as early as 15 weeks of gestation and then increase through gestation, reaching maximum levels of 1–10 ng/ml at term[472] (Figure 42.8). A binding protein (BP) for CRH also exists, and for most of pregnancy it is present in excess of CRH in the maternal circulation.[473–475] As the CRH-BP binds CRH with greater affinity than the CRH receptor, it effectively suppresses CRH bioactivity. Thus, for most of pregnancy, the bulk of the placental CRH is thought to be sequestered by the CRH-BP, limiting its bioavailability. However, during the last 4 weeks of pregnancy, CRH-BP levels decrease markedly.[476–478] This coincides with the exponential increase in placental CRH production, which could result in a dramatic increase in CRH biological activity.[476]

Studies of maternal CRH levels during human pregnancy suggest that CRH produced by the placenta plays a role in the physiology of parturition. The level of CRH in the maternal circulation increases exponentially as gestation progresses, and the pattern of change in maternal CRH levels at midgestation is predictive of the gestation length[476] (Figure 42.8). The rate of CRH increase is inversely related to the length of gestation, such that the incidence of preterm birth is greater in women with a rapid rate of increase in maternal CRH at midgestation, whereas postterm birth is more common in those with a slow rate of increase at midgestation.[476] This effect may be related to the bioavailability of CRH as the CRH-BP levels fall during late pregnancy. Thus, the ratio of CRH to its binding protein may have a threshold level that is achieved at specific times depending on the trajectory of placental CRH expression established early in gestation.

CRH may affect myometrial contractility. Receptors for CRH have been identified in the human myometrium and fetal membranes,[479–481] and in vitro studies have shown that CRH stimulates the release of PGs from human decidua and amnion,[482,483] and augments the action of OT and $PGF_{2\alpha}$ on myometrial contractility.[484–486] During most of pregnancy, CRH increases adenylate cyclase activity in the myometrium, leading to an increase in intracellular cAMP, which promotes relaxation. At the time of parturition, and especially during active labor, the capacity for CRH to increase cAMP decreases.[487] Thus, for most of pregnancy, CRH may contribute to the maintenance of myometrial relaxation, and this effect appears to decrease at parturition.

One important characteristic of placental CRH expression is that it is increased by glucocorticoids,[488–490] an effect that is opposite to hypothalamic CRH expression, which is decreased by glucocorticoids. Stimulation of placental CRH production by glucocorticoids may result in a positive-feedback loop whereby placental CRH stimulates ACTH production by the fetal pituitary, which would increase cortisol and DHEA-S secretion by the fetal adrenals. Fetal adrenal cortisol could then further stimulate placental CRH production, and the DHEA-S would serve as substrate for placental estrogen synthesis. The marked rise in placental CRH during the last 10 weeks of pregnancy could be due to such a positive-feedback interaction, especially as the capacity for the fetal adrenal cortex to synthesize cortisol develops.[325] Interestingly, CRH also may influence fetal adrenal steroidogenesis directly by increasing DHEA-S production[491–493] and ACTH

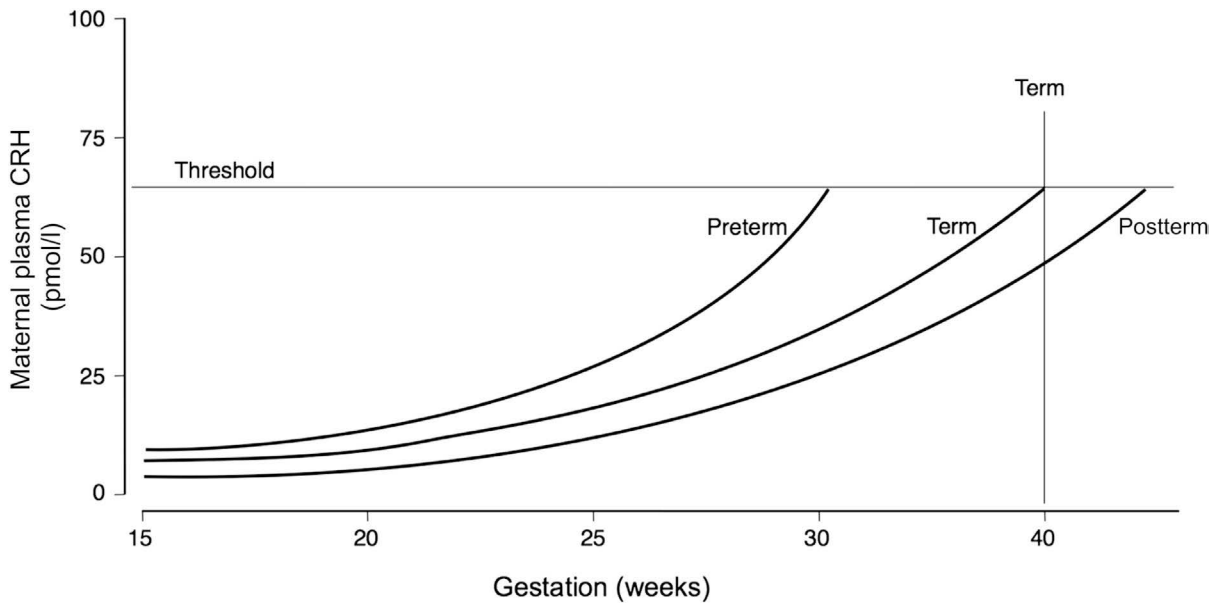

FIGURE 42.8 Schematic representation of maternal plasma CRH levels between 15 and 30 weeks of gestation in pregnancies destined to deliver preterm, at term, and postterm. A theoretical threshold for CRH-induced parturition is shown. The trajectory of CRH increase established early in gestation will influence when the threshold is reached. *Source: From Ref. 476.*

receptor expression in fetal adrenal cortical cells.[494] Thus, a positive-feedback endocrine loop may develop between placental CRH and the fetal adrenal cortex as a consequence of the direct action of CRH on the fetal adrenal cortex and the stimulation of placental CRH expression by cortisol. The effect of this endocrine axis on the physiology of human parturition is not clearly defined; however, it may represent a link between the fetal-placental stress response and parturition. Some investigators have suggested that the complex functional interaction between placental CRH and the fetal HPA axis represents a potential link between the putative placental clock, reflected by placental CRH, and the production of glucocorticoids needed for fetal organ maturation and estrogens needed for the process of parturition.[476]

Biomechanical Influences

Uterine distention has been proposed as a signal for the induction of parturition to ensure that the fetus does not grow larger than the pelvic opening. The human uterus is subjected to a significant amount of distention, especially late in the third trimester and in pregnancies with multiple fetuses. A likely scenario is that a threshold of uterine wall distention exists above which the induction of pro-labor and proinflammatory gene expression contributes to the parturition cascade.

Clinical evidence that uterine wall distention contributes to the parturition process is that gestation is generally shorter in pregnancies with multiple fetuses.[495,496] Studies in rats have shown that distention of a nonpregnant uterine horn induces changes in the expression of genes associated with contraction and parturition similar to those in the pregnant horn, and that the parturition-related changes in gene expression are inhibited by progesterone.[39,40,497–500] The stimulatory effect of distention was also observed in cultured human myometrial cells.[388,501–504] Stretch of cultured uterine myocytes also increased the expression and release of IL8, a potent proinflammatory chemokine that promotes the infiltration of activated macrophage and neutrophil infiltration.[502] Thus, myometrial cell distention could contribute to the positive-feedback proinflammatory loop that leads to tissue-level inflammation and the onset of labor. Similar mechanotransduction signaling pathways could be operating in the cervix, especially as it ripens and becomes more distensible.

Signals from the Fetus

At birth, the fetus is abruptly required to establish and maintain physiologic homeostasis independent of the placenta and in a markedly altered environment. Survival of the neonate is therefore dependent upon the functional maturation of organ systems during fetal life that will be essential for extrauterine life. Critical among these are organs that interface with the environment (e.g., the lungs, gut, and immune system) and those that maintain homeostasis (e.g., the HPA axis, kidneys, liver, and pancreas). The coordination of organ maturation and the timing of parturition such that birth occurs when the fetus is sufficiently mature to survive as a newborn is therefore critical for the success of pregnancy.

In most species, glucocorticoid produced by the fetal HPA axis promotes the functional maturation of fetal organ systems and coordinates fetal maturation with birth timing.[248,505] In sheep, fetal organ maturation is induced by a prepartum surge in cortisol secretion by the fetal adrenals.[505] The cortisol surge also triggers the onset of labor. Thus, in sheep, the fetal HPA axis, via cortisol, mediates a physiologic link between the timing of birth and fetal organ maturation. A prepartum fetal cortisol surge does not occur in human parturition, and the fetal HPA axis plays a minor role (if any) in the control of human parturition. Nonetheless, glucocorticoid is a key stimulator of fetal organ maturation in humans. Critical processes induced by glucocorticoid include surfactant production by the fetal lungs; activity of enzyme systems in the fetal gut, retina, pancreas, thyroid gland, and brain; and deposition of glycogen in the fetal liver. Synthetic glucocorticoids that readily cross the placenta are a standard form of therapy administered to women in preterm labor.[506] This treatment significantly increases survival rates among preterm infants, mainly by promoting lung maturation and decreasing the severity of respiratory distress syndrome.

The extent to which cortisol from the fetal adrenals regulates fetal organ maturation in human pregnancy is uncertain. Experiments of nature suggest that maturation of the human fetus during late gestation is independent of fetal adrenal cortisol production. Fetuses with congenital adrenal hyperplasia (CAH) due to an inability to synthesize cortisol are usually born at term without any apparent signs of organ immaturity.[255] This observation suggests that cortisol produced by the fetal adrenal gland is not essential for fetal organ maturation; another source of glucocorticoid, possibly from the maternal adrenals, could contribute to fetal organ maturation at the end of human gestation; or maturation of the human fetus is not dependent upon glucocorticoids alone and other factors may be involved.

Although cortisol stimulates maturation of fetal organ systems, it also can have adverse effects on fetal development. To protect the fetus from these negative effects, the human placenta prevents maternal cortisol from entering the fetal compartment throughout most of human pregnancy by converting cortisol to the inactive cortisone.[507] Late in human pregnancy (around 34–35 weeks), however, the placental barrier to maternal

cortisol weakens. The evidence for this is that estriol levels in the maternal circulation during late pregnancy are inversely related to the circadian changes in circulating maternal cortisol levels.[508] Thus, when maternal cortisol goes up, estriol goes down. This implies that, late in gestation, some maternal cortisol crosses the placenta to the fetal compartment and suppresses ACTH production by the fetal pituitary gland that leads to decreased DHEA-S production by the fetal adrenals and, in turn, decreased estriol production by the placenta. Increased transfer of maternal cortisol to the fetus may represent a backup mechanism to ensure fetal organ maturation. This transfer of maternal cortisol, rather than lack of dependence on cortisol, may explain why fetuses with glucocorticoid deficiency are born without overt signs of organ system immaturity.

The fetus may trigger parturition as an adaptation to escape intrauterine stress caused by pathophysiologic conditions such as preeclampsia, intrauterine growth retardation, nutritional stress and increased uterine wall distention due to multiple fetuses, and intrauterine infection, all of which are associated with preterm birth.[509,510] The physiologic link between fetal stress and parturition may be via placental CRH. Placental CRH production is increased in cases of intrauterine infection and hypoxia.[511] Whatever the mechanism, it is clear that fetal stress is somehow linked to the parturition trigger mechanism. This is an important clinical issue because, in some cases of preterm birth, the fetus may be escaping a hostile intrauterine environment, and therefore prevention of preterm birth in those cases may be detrimental to the fetus if the cause of the stress is not treated.

PATHOPHYSIOLOGY OF HUMAN PARTURITION

Preterm Birth

Understanding the mechanisms coupling the rate of fetal maturation with the timing for birth provides a fundamental area for biological investigation that is as important for mammalian survival as the sequence of events producing a differentiated multicellular organism from a single-cell embryo. Beyond this intrinsic scientific interest, elucidation of the determinants of the onset of human parturition will advance interventions for perhaps the most challenging, and refractory, syndrome in women's and children's health, preterm birth.[512] Human preterm birth, defined as birth at less than 37 completed weeks of gestation, results in 75% of perinatal mortality and the majority of long-term perinatal morbidity.[513] While the World Health Organization definition of preterm birth at less than 37 weeks is somewhat arbitrary, it is clear that birth at every gestational age before term, including in the 37th and 38th weeks of gestation, is associated with increased perinatal morbidity and neonatal mortality.[514,515] Approximately 75% of preterm births occur between 34 and 36 weeks of gestation, which are designated "late-preterm" births. The increase in frequency of delivery rises exponentially as term approaches, such that approximately 5% of preterm births, or less than 1% of total births, occur at less than 28 weeks of completed gestation, when fetal mortality and morbidity are highest.[208] The impact of late-preterm births should not be underestimated, however, as a three- to sixfold increase in neonatal mortality has been found for infants born at these gestational ages.[516]

Several recent comprehensive review articles exist regarding the epidemiology of human preterm birth.[208,512,517,518] The conceptual framework for parsing the etiology of preterm birth first begins with dichotomizing the pattern of delivery as "spontaneous" or "medically indicated". Medically indicated deliveries account for approximately 30% of preterm births. The vast majority of medically indicated deliveries occur in the late-preterm gestational age window and are initiated because of the appearance of maternal or fetal abnormalities that could ultimately compromise healthy pregnancy outcomes, including maternal or fetal death. One frequent antecedent of medically indicated deliveries is preeclampsia.[519,520] The diagnosis of preeclampsia encompasses increased maternal blood pressure (systolic > 140 mm Hg or diastolic > 90 mm Hg) with proteinuria (>300 mg protein per 24 h urine collection). A spectrum of severity for preeclampsia exists, with additional manifestations including maternal cerebral or visual disturbances, pulmonary edema, liver function abnormalities, and thrombocytopenia, and fetal growth restriction. Importantly, this disorder, which manifests structural vascular changes in the maternal kidney and other organs, with compromised tissue perfusion due to vasospasm, resolves with delivery of the placenta.[521–523] A complete discussion of the pathophysiology and etiology of preeclampsia is beyond the scope of this chapter, but can be found in several excellent reviews.[519,524] The remainder of the discussion on preterm birth will focus on causes of spontaneous preterm birth that could arise from either dysregulation of typical parturition control mechanisms or distinct pathological pathways.

Among the 70% of preterm births ascribed as being spontaneous, approximately two-thirds are due to spontaneous preterm labor and one-third arise from PPROM.[208] In the United States, 11.7% of births are preterm based upon 2011 data.[525] This rate is 15% higher than the preterm birth rate in 1990, although it reflects a consistent decline from its peak in 2006 at 12.8%.[526] The etiology of the decline in preterm births in the United States stems largely from changes in obstetric practice for labor induction or Cesarean section in the late-preterm

category.[208,517,525,527,528] Births at less than 34 weeks of completed gestation, in contrast, have not changed substantially in frequency over the last 20 years. The direct causal pathways for spontaneous preterm birth are unknown in any given pregnancy in more than half of the cases.[208,518] Multiple gestations, arising either naturally or as a consequence of assisted reproductive technology, complicate 2–3% of pregnancies but result in 15% of all preterm births. While the traditional mechanism for early births in twin or higher multiplicity pregnancies has been attributed to uterine overdistention, recent evidence measuring uterine wall strain calls this model into question.[529]

For singleton gestation pregnancies, several maternal demographic characteristics have been associated with increased preterm birth risk. These include low maternal body mass index (BMI), extremes of maternal age, African-American race, low socioeconomic status, smoking, lack of prenatal care, short interpregnancy interval, and previous preterm birth.[208,530–532] How these characteristics disrupt the normal timing for birth remains uncertain. In addition to these maternal demographic factors, strong evidence provides causal links of genetics, infection, and stress to preterm birth risk.[518,533–539]

Genetics and preterm birth risk: Several lines of evidence converge to implicate the importance of genetic factors in the mother or fetus as influencing the duration of gestation and preterm birth risk. Studies exploring familial patterns for birth timing have been particularly informative. Large-scale epidemiological analyses from European populations demonstrate a significantly increased risk of preterm birth in sisters of women who have experienced a preterm delivery, but little or no increase in risk to women who are sisters of fathers who have had a preterm child.[540,541] Analysis of birth timing in children born to monozygotic twins, in comparison with dizygotic twins or nontwin siblings, supports an important role for maternal genetics in the determination of gestational age at birth, with heritability estimates ranging from 14% to 40%.[542–545] The role of paternal genes has not been as extensively investigated, but studies suggest a much smaller role based on mixed-race pregnancies[546] or no role based on children of twin-type analysis.[543] The role of fetal genes in parturition has recently received considerable attention. Elegant family-based and twin-modeling investigations have demonstrated an important role for fetal genetics, contributing approximately 11% of the variability in birth timing,[545,547] which is supported by segregation analysis in family pedigrees.[548]

Armed with this evidence for genetic contributors to human birth timing, the hunt for human genomic variations that contribute to preterm birth risk has gained considerable attention. Initial interrogation of the role of gene polymorphisms exploited candidate gene approaches, exploring pathways that have been implicated in parturition such as inflammation and immunity, connective tissue remodeling, hemostasis, and control of myometrial contractility and quiescence. While individual studies have found small to moderate associations with variants in cytokines, their receptors, MMPs, coagulation factors, and adrenergic receptors, for example, these have in general not been replicated or generalized. Several excellent summaries of candidate gene studies in preterm birth have recently been published.[537,549,550] Nonbiased, genome-wide association studies and whole-exome sequencing are currently underway, but to date, novel common or rare genetic variants lending new insight into parturition pathways have not been reported.

Infection and preterm birth risk: Clinical, histological, and microbiological evidence suggests that infection may contribute to 25–40% of preterm births.[208,462,539] Bacterial colonization of the placenta or amniotic fluid, particularly with *Ureaplasma urealyticum*, as detected by culture or polymerase chain reaction, is found in 79% of pregnancies with birth at 23 weeks of gestation and declines to 11% in pregnancies with birth at 31–34 weeks of gestation.[551] Intriguingly, in women found to be colonized in amniotic fluid with *Ureaplasma* at midgestation, only 30% go on to deliver preterm.[552,553] Histologic evidence of both maternal and fetal inflammatory responses similarly increases in frequency in what have otherwise been deemed idiopathic spontaneous preterm births as gestational age at birth diminishes.[554] Together, these findings suggest that infection may contribute most prominently to very early gestation preterm birth. Unfortunately, pregnant women with vaginal colonization with *Ureaplasma* or *Mycoplasma* receiving prophylactic treatment with antibiotics do not experience a reduction in the preterm birth rate.[555]

More recent concepts regarding the role of bacterial pathogens as contributors to preterm birth risk consider, rather than a primary microorganism, a shift in the microbial community as the driver of adverse pregnancy outcomes.[556–559] One such example is bacterial vaginosis. Analogous to results from attempts to eradicate *Ureaplasma*, treatment for bacterial vaginosis,[560] or other chronic, low-grade infections such as trichomoniasis[561] and periodontal disease,[562] has not reduced preterm birth risk. Ongoing attempts to more fully characterize the full microbial community—encompassing bacteria, fungi, and viruses—defining the metagenome of pregnancy may reveal new, previously cryptic pathogens that would not have been eradicated by previous antibiotic interventions.

Stress and preterm birth risk: Many maternal demographic factors that increase risk for preterm birth directly or indirectly associate with the frequency and severity of physiological or psychosocial stress that mothers experience.[208,518,536,563,564] Examples of these demographic factors include living in poverty, low

maternal BMI, unmarried status, and racial minority status. As originally described by Selye, deviation of an organism from its homeostatic set point (i.e., stress) initiates a highly conserved endocrine and sympathetic nervous response to promote adaptation and return to homeostasis.[565,566] The primary endocrine response centers on the synthesis and secretion of glucocorticoids, cortisol in humans and corticosterone in rodents, from the adrenal. Glucocorticoids exert a panoply of effects on metabolism, immune function, and cognition that promote adaptation acutely, but compromise well-being when present chronically.[567,568] Glucocorticoids independently play a critical role in fetal maturation and the timing for parturition in many species. In humans, the rise in glucocorticoids late in typical term pregnancy is thought to promote fetal lung, and other organ, maturation in preparation for delivery, and accelerate the labor process by inducing PG synthesis by the fetal membranes.[186–188,569,570] Prematurely, chronically elevated glucocorticoids associated with stress during pregnancy are hypothesized to induce PGs prior to term, and cause vascular, cervical, and myometrial responses that promote preterm delivery. Consistent with this notion, in otherwise uncomplicated pregnancies, high psychosocial stress was associated with significantly increased biomarkers of inflammation, such as C-reactive protein and a shorter cervix late in gestation.[571] Additionally, women with higher indices of anxiety according to the Spielberger questionnaire demonstrated increased mean uterine artery resistance index,[572] a parameter associated with a higher rate of preterm birth.[573]

Progestin Therapy to Prevent Preterm Birth

The idea of progesterone therapy to control parturition, and especially to prevent preterm labor, was proposed in the 1940s, but it was not until the late 1950s that sufficient amounts of progesterone became available for clinical studies. Small trials in the 1950s and 1960s suggested that administration of large amounts of progesterone could be used repress the contractions of labor.[297,298] However, more thorough studies in the 1970s and 1980s produced mixed and conflicting results, and consequently the approach was abandoned because its effectiveness was questionable. In addition to the conflicting clinical data, the approach was, and to date still is, burdened by the biological conundrum of how further progestin supplementation of already high endogenous progesterone levels could have effects. Nonetheless, several meta-analyses of early studies rekindled interest by showing that prophylactic progestin therapy (rather than therapy at the time of labor) may reduce the incidence of preterm birth.[574–576] Studies were therefore conducted to assess the effectiveness of prophylactic treatment with natural progesterone administered vaginally or a caproate ester of 17α-hydroxyprogesterone (17HPC) given as long-acting intramuscular injections beginning at around midgestation.[577,578] Taken together, those trials showed that prophylactic progestin therapy decreases the incidence of preterm birth and improves neonatal outcome, but only in women with an increased risk for preterm birth (based on a prior preterm birth). Subsequent studies showed that the therapy was also effective in women with a short cervix detected by ultrasound at midgestation, which is a strong predictor for preterm birth.[95,579] Interestingly, da Fonseca[577] reported that progesterone via vaginal suppositories did not change the incidence of women presenting with preterm labor but increased the effectiveness of tocolytic treatment with beta-mimetics to prevent preterm birth compared with women in the placebo group. This effect is consistent with nongenomic actions of progesterone reported in myometrial strips,[303] wherein progesterone decreases contractility and augments the capacity for beta-mimetics to block OT-induced contractions. Prophylactic vaginal administration of progesterone, however, failed to alter preterm birth rates in the general population, and administration of 17HPC to women with twin pregnancies (who generally deliver preterm) did not decrease the incidence of preterm birth.[580,581] The reason why progestin therapy is effective in only a small subset of at-risk pregnancies is unclear, and the value of prophylactic progestin therapy to prevent preterm birth in the general population is yet to be established.[582]

Animal models of preterm birth

Defining causal mechanisms for preterm birth has necessitated the establishment of informative animal models, as experimental manipulation and analysis of human pregnancy are not possible with existing technologies. As with using nonprimate models for the elucidation of term pregnancy, divergence in endocrine control mechanisms for the maintenance and termination of pregnancy provides similar challenges to extrapolating model organism findings to human pregnancy.[6,7] This limitation once again revolves around the overt progesterone withdrawal associated with labor, preterm or term, in ruminants and rodents, but missing in human term and preterm labor. Nonetheless, important observations from the animal studies will facilitate developing new mechanistic hypotheses for humans. Several species and experimental paradigms have been developed to understand mechanisms for preterm birth. Here, we will summarize key findings from three species: sheep, mice, and nonhuman primates.

Sheep: Elegant physiological studies in sheep have established premature activation of the fetal hypothalamic–pituitary–adrenal axis, or exposure to exogenous glucocorticoids, as drivers of parturition at

term or preterm.[3–5] Pioneering studies by Liggins and colleagues demonstrated that fetal infusion with ACTH or cortisol initiated a shift from progesterone to estradiol synthesis by the ovine placenta, and initiated preterm birth.[4] This exogenous exposure, resulting in fetal adrenal activation or directly providing the product of fetal adrenal activation, cortisol, mimics the normal-term parturition pathway in sheep. Glucocorticoid administration to pregnant women, as routinely occurs in those with threatened preterm labor, to accelerate lung maturation of the fetus does not result in preterm labor. This finding demonstrates that glucocorticoids, while accelerating human fetal organ maturation, do not exert the same effects for parturition initiation in humans and sheep. In rodents, glucocorticoid administration tends to delay the onset of parturition.[583]

Beyond the role of glucocorticoids in precipitating parturition in sheep, ovine pregnancy has provided an important model for understanding the effects of nutrition on preterm birth risk. An important study by Bloomfield and colleagues[584] investigated the role of periconceptual undernutrition by comparing the timing of birth in well-fed ewes to those that had undergone moderate undernutrition from 60 days before conception through 30 days following conception (15% maternal weight loss). This limited period of undernutrition resulted in a significantly lower mean gestational age at birth in the restricted-feeding group (139 days) in comparison to the typically fed animals (146 days; $p < 0.05$). The ramifications for human pregnancy, particularly related to low maternal BMI, are intriguing. The mechanism for the early labor in the sheep was premature activation of the HPA axis.[585,586] This mechanism is unlikely to be responsible for early birth in human undernourished pregnancies, but precocious activation of the normal human pathway for labor may similarly result.

Mice: With the advent of transgenic and gene knockout technologies, mice provide a tractable system for investigating the mechanisms by which developmental pathways and environmental exposures determine gestation length. The caveats that the mouse provides for investigating term partition mechanisms—luteal progesterone production, multifetal pregnancy, and overt progesterone withdrawal at parturition—similarly confound the extrapolation of these mechanisms for preterm birth in this species to human pregnancy. Recently, however, models of preterm birth in mice without overt progesterone withdrawal have been reported.

Gene ablation studies in mice have most often generated lines that fail to initiate parturition at term. More recently, however, several interesting gene-targeting studies have produced strains that undergo spontaneous preterm birth. Because of human data demonstrating the downregulation of HPDG in the chorion of women experiencing preterm labor and delivery, Roizen and colleagues generated mice with a hypomorphic allele of HPGD, as complete inactivation of HPGD in female mice results in infertility (Roizen and Muglia, unpublished data). Matings of HPGD hypomorphic males and females resulted in pregnancies that delivered approximately 1 day preterm on average.[406] Matings of wild-type males with HPGD hypomorphic females resulted in pregnancies that did not significantly differ from pregnancies in wild-type females mated to wild-type males. This finding suggests an important role for fetal HPGD in regulating the timing for birth in mice, in the same compartment of the fetal–maternal interface as has been implicated in humans. Furthermore, the preterm birth in HPGD pregnancies was not associated with progesterone withdrawal, indicating that one genetic manipulation can shift the pattern of endocrine regulation of labor from a progesterone-withdrawal to a nonprogesterone-withdrawal phenotype.[406] The pregnancy characteristics of the HPDG hypomorphic line also demonstrated a relative predominance of PGE_2, as compared with $PGF_{2\alpha}$, in accord with human pregnancy. Dey and colleagues reported the generation of mice with uterine-specific disruption of the tumor suppressor p53.[587,588] These mice demonstrate accelerated uterine decidual senescence, activation of the target of rapamycin complex 1 (mTORC1) signaling, and preterm birth. This uterine p53 deletion model provides a new paradigm for antecedents of preterm birth that are established early in pregnancy and incur later consequences for birth timing. Mice with deletion of the cannabinoid receptor type 1 also manifest preterm labor (approximately one-half day early), but this appears to be due to accelerated luteolysis.[589] Most recently, uterine deletion of the transforming growth factor beta superfamily member, Nodal, resulted in mice with pregnancy termination predominantly at day 17.5 of gestation (term 19.5 days) that resulted from aberrant placentation and a diminished decidua basalis.[590] Maternal serum progesterone concentration was reduced at day 16.5 compared to controls, but remained at a level above that usually associated with luteolysis-mediated term delivery in mice. Whether the Nodal model reflects progesterone withdrawal or not remains to be determined.

Several infection and inflammation models have been developed in rodents, and they have been systematically reviewed in Elovitz and Mrinlani.[591] Initially, investigations in mice explored the ability of the TLR4 ligand lipopolysaccharide (LPS), which was administered systemically, to cause preterm labor. Given at day 14.5–15.5 of gestation, LPS typically results in preterm labor within 24 h in gravid female mice.[419,592,593] The LPS exposure is associated with induction of uterine PTGS2 expression, enhanced generation of $PGF_{2\alpha}$, and overt progesterone withdrawal.[594] In contrast, the use of

heat-killed *Escherichia coli* (HKE) administered by intrauterine injection caused preterm labor without progesterone withdrawal.[460] It is likely that the differing routes of administration, with more restricted local effects with intrauterine administration, yield these differences for progesterone regulation. Given the ability to explore individual components of the signaling cascade induced by bacterial exposure, several studies have dissected the role for individual cytokines and receptors in this process. While, as expected, maternal TLR4 is required for preterm birth due to HKE exposure,[595] loss of individual cytokines in genetically ablated mice, such as IL1β, the IL1 receptor, and IL6, did not protect from preterm birth.[596–598] Loss of both IL1R1 and TNF receptor superfamily member 1A in mice did demonstrate some protection from preterm birth with HKE, suggesting redundancy in cytokine signaling pathways.[599]

To investigate the signals potentially coordinating fetal maturation with the timing for birth, Mendelson and colleagues explored the role of the pulmonary surfactant protein A (SPA).[600] SPA not only serves to promote pulmonary function but also may signal to activate macrophages to initiate a proinflammatory response. Intraamniotic injection of SPA during mouse pregnancy prior to its normal induction during fetal lung development caused preterm labor within 6–24 h of administration.[600] Conversely, injection of an antibody to SPA in normal pregnancies delayed the time to the onset of labor.[600] The intraamniotic injection of SPA was associated with migration of fetal macrophages into the myometrium and activation of IL1β and NFκB. These actions are anticipated to generate downstream signals directly leading to uterine contractions. Mice deficient in SPA or SPA and SPD have normal onset of parturition in their first pregnancy, but exhibit an approximately 12 h delay in later pregnancies.[601] Mice deficient in TLR2, which is believed to be activated by SPA, also manifest a mild delay in the onset of parturition.[601] Whether similar actions occur in human pregnancy is uncertain. In laboring women, macrophages of fetal origin have not been detected in the myometrium, and fetal SPA levels are robustly elevated several weeks prior to the onset of term labor in humans.[602,603]

Nonhuman primates: Nonhuman primates, particularly rhesus macaques, have been important models for preterm birth based upon their similar physiology to human pregnancy. Perhaps the most informative studies related to preterm birth in these nonhuman primates evaluate inflammatory and infectious contributions to preterm birth. For example, Novy and colleagues developed a very useful chronically catheterized model system in rhesus monkeys that allows serial sampling of maternal and fetal compartments for hormone and cytokine changes associated with preterm birth.[408,459] They demonstrated that acute administration of IL1β or TNFα in the amniotic fluid during rhesus pregnancy at 80% of gestation (130 days) initiated a sustained cytokine cascade, with endogenous production of TNFα and IL1β, and preterm contractions and delivery.[459] Intraamniotic administration of *Ureaplasma* at a similar time during gestation likewise initiated a fetal and maternal cytokine response, and preterm delivery.[604] Curiously, the sheep does not initiate preterm labor and delivery with intraamniotic *Ureaplasma* administration.[605] Thus, the rhesus provides a necessary system for mechanistic insights, including the identification of biomarkers that may more accurately predict the likelihood of human preterm birth associated with *Ureaplasma* colonization.

Decidual Hemorrhage

Vaginal bleeding during early pregnancy is an important risk factor for preterm birth. Bleeding in the first trimester is associated with a twofold increase in rate of preterm birth (95% confidence interval (CI): 1.6–2.5), while if bleeding is present in more than one trimester, the odds ratio for PPROM increases to 7.4 (95% CI: 2.2–25.6).[606,607] The most likely sites of bleeding arise between the decidua parietalis and the chorion, or the basal plate of the placenta and the decidua (abruption placenta). Vascular lesions in the decidual vessels adjacent to the placenta have been reported in one-third of women with preterm labor and intact membranes or PPROM, but only one-eighth of women delivering at term.[608–610] The biochemical mechanisms leading to preterm labor and membrane rupture with decidual hemorrhage include augmented thrombin generation.[611,612] The decidua is an abundant source of tissue factor, a strong initiator of the coagulation cascade. Hemorrhage involving the decidua would then generate large quantities of thrombin. Thrombin has been found to stimulate uterine contractions in a dose-dependent manner, and it also stimulates the production of MMP1, urokinase-type plasminogen activator, and tissue-type plasminogen activator by decidualized endometrial stromal cells in culture, and MMP3 in the decidua, which degrades local ECM components and further serves to induce MMP1 and MMP9 in the fetal membranes.[613–615] Thrombin additionally activates cytokine production, such as IL8 by decidual cells, a potent neutrophil chemoattractant, and IL11, an inducer of PGE_2.[611,616]

Postterm Birth and Failure to Progress

Postterm birth is defined as delivery after 42 weeks of completed gestation. The rate of postterm birth ranges from 3% to 14%, depending upon the population studied.[617–619] Like preterm birth, postterm birth is associated with significant adverse sequelae for both the mother and the infant, including Cesarean

delivery, postpartum hemorrhage, shoulder dystocia, fetal hypoxic–ischemic injury, and meconium aspiration. Prolonged pregnancy (i.e., lasting more than 41 weeks of completed gestation) is associated with increased risk for the same detrimental outcomes.[620,621] Several maternal risk factors have been associated with postterm pregnancy. These include nulliparity, obesity or high (>35 kg/m^2) BMI, and previous postterm birth.[618,621,622] Black race is associated with a decreased frequency of postterm birth.[618] The recurrence of postterm birth in the same mother suggests a potential role for genetic contributors, as has been suggested by several studies. In addition, the association of placental sulfatase deficiency to prolonged pregnancy risk, by limiting late gestation rises in estrogen production, further supports the involvement of genetic factors to postterm delivery.[623] The mechanisms by which maternal BMI and nulliparity increase the risk for postterm birth remain unclear. Alterations in the endocrine and metabolic milieux of pregnancy resulting from both of these influences have been postulated, but direct evidence has not yet been obtained.

Animal models of postterm birth have arisen through both naturally occurring variations and targeted genetic manipulations. In general, small litter size in typically multifetal rodent gestations is associated with prolonged gestation prior to delivery.[624] Often, the consequence of this is excessive fetal size resulting in failure of delivery of the pup after placental detachment.[625] This pregnancy outcome, which can result in death of the dam and pups, would be strongly selected against during evolution. As a reflection of this evolutionary pressure, small numbers of implanted embryos early in gestation are often inadequate to sustain pregnancy. Several mouse genetic models are associated with postterm pregnancy or complete inability to initiate parturition. The largest functional category of mutations yielding this outcome in mice is that associated with PG, specifically $PGF_{2\alpha}$, action in late gestation. Mutations in cPLA2 and PTGS1 result in impaired $PGF_{2\alpha}$ synthesis, and mutations in the FP receptor result in defective $PGF_{2\alpha}$ signaling and lead to delayed or absent partition.[403,405,626,627] The target for control of parturition in these situations is on the maternal ovarian CL to cause luteolysis. Direct uterotonic actions of $PGF_{2\alpha}$ appear dispensable, as ovariectomy rescues the normal onset and progression of labor in FP knockout mice.[403] The redundant actions of other PGs and OT likely compensate for loss of FP action in the myometrium. Mutations in 20-α hydroxysteroid dehydrogenase can similarly result in prolonged gestation by limiting the conversion of progesterone to biologically inactive metabolites.[628] Genetic ablation of steroid 5-α reductase type I (SRD5α1) also leads to late parturition in mice.[282,629] In this circumstance, luteolysis occurs at the normal time. However, prolonged actions of progesterone on the cervix, due to impaired local metabolism by SRD5α1, lead to a failure of cervical ripening and pup delivery.[282,629] Recently, mutations in the transcription factor Kruppel-like factor 9 have been associated with prolonged gestation in mice.[630] The mechanism for this abnormal parturition may stem from dysregulated PR isoform switching late in gestation. To date, the involvement of none of these mechanisms has been associated with postterm human gestation.

Uterine Atony

During late pregnancy, the placental bed receives approximately 800 ml/min of blood flow.[631,632] With detachment and delivery of the placenta, cessation of this high rate of blood flow must occur to prevent postpartum hemorrhage. The mechanism by which hemorrhage is prevented is compression of the uterine vasculature by contraction and retraction of myometrial fibers. Failure to achieve this uterine contraction, uterine atony, results in significant morbidity and mortality in many parts of the world where access to uterine contractile agents is not available.[633] Risk factors for uterine atony include uterine overdistention due to multifetal pregnancy, polyhydramnios and fetal macrosomia, exposure to uterine relaxants such as magnesium sulfate, prolonged labor, induction or augmentation of labor, manual removal of the placenta, obesity, advanced maternal age, and previous postpartum hemorrhage.[634,635] To prevent or treat uterine atony, uterine massage and injection of OT are first-line therapies.[633] The administration of ergometrine or methylergonovine also has proven efficacious. These agents cause prolonged tonic uterine contraction by activating smooth muscle α-adrenergic receptors. Atony that is refractory to OT and ergot alkaloids necessitates further intervention by PG administration, typically 15-methyl $PGF_{2\alpha}$ or misoprostol, and derivatives of prostaglandin E1.[633] Hemostatic agents such as activated factor VII or tranexamic acid, or interventions such as uterine tamponade, are additional adjunctive therapies that can be applied.[636–638]

CLINICAL CONTROL OF PARTURITION: TOCOLYTICS AND INDUCTION OF LABOR

Women who develop preterm labor may be candidates for tocolytic therapy. The foundation for tocolytic agents centers on their ability to block the physiological pathways described for myometrial activation during parturition. Tocolytic agents are typically administered after the onset of preterm parturition is clinically apparent. The goal of therapy is to inhibit uterine contractility

and ultimately achieve uterine quiescence in an effort to prolong gestation. There are a number of tocolytic agents that are currently used in the management of preterm labor in contemporary practice, although none is clearly superior to the others. There is no single agent currently considered to be the first-line tocolytic medication.[639] All currently available tocolytic agents have relatively limited efficacy, with the ability to delay delivery for only a short period of time (ranging from a few days to a week), likely due to redundant uterine contraction activation mechanisms.

Women who present with preterm labor between the gestational ages of 24 and 34 weeks may be considered candidates for tocolytic therapy. Tocolytic therapy is recommended only within this gestational age window because this is when antenatal corticosteroid therapy has proven efficacious to reduce neonatal morbidity.[640,641] There are some uncommon circumstances in which some practitioners may feel that inhibition of contractions at previable gestational ages may prevent serious pregnancy complications, or that it may be of use for women who develop late-preterm labor (34–36 weeks) in an effort to stabilize the pregnancy long enough to transport the patient to a tertiary care facility before delivery.

The most commonly used tocolytic agents inhibit uterine contractions through decreasing the availability of calcium or inhibiting its transfer into myometrial cells (magnesium sulfate, beta-mimetics, and calcium channel blockers). Others attempt to inhibit uterine myometrial contractions through blocking uterotonins such as PGs or oxytocin receptor (oxytocin receptor antagonists).

Calcium Channel Blockers

Calcium channel blockers are nonspecific smooth muscle relaxants. They act on L-type calcium channels, which are widely expressed in cardiac and smooth muscle cells, by inhibiting the slow influx of extracellular calcium into the muscle.[642] They were originally, and remain most commonly, used for their vasodilatory effect in the treatment of hypertension. Calcium channel blockers have a much more potent relaxant effect on smooth muscle as compared to their direct effect on myocardium, with only minimal negative inotropism.[643] Their antagonistic effect on slow calcium current into the muscle results in low intracellular calcium and smooth muscle relaxation. Their potent smooth muscle relaxant effect on the uterus has more recently led to their use as a tocolytic agent in an effort to inhibit myometrial contractility in cases of preterm labor. The most commonly used calcium channel blocker for tocolysis of preterm labor is nifedipine, which is widely used and often considered the preferred first-line agent. Although it is a nonspecific smooth muscle relaxant, it has demonstrated a potent myometrial relaxant effect, which is superior to other tocolytic agents studied on human myometrial cells in vitro.[644]

Magnesium Sulfate

Magnesium sulfate was until recently the most frequently used tocolytic agent for treatment of preterm labor.[645] Following harsh criticism in the obstetric literature regarding its lack of proven efficacy and concerns regarding its high risk of adverse side effects, many obstetric practitioners have begun to use calcium channel blockers rather than magnesium sulfate for first-line treatment of preterm labor.[646] The mechanism of action of magnesium sulfate is not as well described as that of calcium channel blockers, but it appears to function in a similar manner by competitively blocking intracellular calcium channels, decreasing calcium availability and thus inhibiting smooth muscle contractility.[647] It also competes with calcium at the motor end plate, where it reduces excitation by inhibiting acetyl choline release.[648] Magnesium sulfate has poor patient tolerance when administered in adequate doses for tocolysis. Due to limited evidence of benefit, maternal risk of toxicity, and poor patient tolerance, the use of magnesium for the treatment of preterm labor is decreasing in recent years in favor of other tocolytic agents.

Prostaglandin Synthase Inhibitors

PG synthesis inhibitors, or more specifically COX inhibitors, block the conversion of arachidonic acid to mature PGs. Indomethacin is the most widely used COX inhibitor used for tocolysis of preterm labor. It is a nonselective COX inhibitor, acting on both PTGS1 and PTGS2 (i.e., COX1 and COX2). Other more selective PTGS2 inhibitors, such as celecoxib and rofecoxib, also have been used for inhibition of preterm labor.

Indomethacin tocolysis is generally well tolerated by the mother with few side effects following a short course of treatment. Indomethacin does cross the placenta and has been associated with a variety of adverse effects in the fetus. Constriction of the ductus arteriosus occurs in up to 50% of fetuses exposed to indomethacin after several days of therapy by inhibiting the formation of prostacyclin and PGE_2, which are necessary to maintain ductal vasodilation.[649] Other fetal and neonatal complications have been reported with use of indomethacin such as oligohydramnios, primary pulmonary hypertension, and necrotizing enterocolitis.[409,650,651] Although a causal link with most severe neonatal complications has

not been clearly identified, concern regarding fetal and neonatal risk with prolonged use of nonsteroidal anti-inflammatory drugs, especially at later gestational ages (>32 weeks), has limited their use to a short duration of no longer than 2 days.

Oxytocin Receptor Antagonists

OT receptors are present in myometrial cells and myoepithelial cells in mammary glands, and relatively limited other peripheral sites. In theory, a specific OTR blocker should be quite effective for inhibition of labor with limited maternal side effects. One OTR blocker, atosiban, has been investigated for the prevention of preterm birth in several studies. A systematic review of randomized trials found that atosiban was not associated with a reduction in the rate of preterm birth or an improvement in maternal or neonatal outcomes. Although it caused fewer adverse maternal drug reactions, it was not found to be superior to other tocolytic agents.[652] In addition, a small but increased rate of infant deaths in the atosiban group identified in this meta-analysis led to safety concerns contributing to the US Food and Drug Administration's (FDA) decision to not approve it for tocolytic use in the United States.[653]

Beta-Adrenergic Receptor Agonists

Beta-sympathomimetics such as terbutaline and ritodrine have been widely used to treat preterm labor for over four decades. They inhibit uterine contractility by binding to β_2 receptors on myometrial cells, activating adenylyl cyclase and PKA and ultimately suppressing myometrial contractility.[653] These beta-adrenergic receptor agonists also stimulate β_1 receptors in the heart, vascular system, and liver, causing significant adverse maternal side effects that limit their tolerability and safety. Common maternal side effects include tachycardia, palpitations, tremor, chest discomfort and dyspnea, and hyperglycemia.[654] In addition, these agents cross the placenta and may cause adverse fetal effects such as fetal tachycardia, neonatal hypoglycemia, hypocalcemia, and ileus. Ritodrine is the only FDA-approved tocolytic medication; however, it is no longer available in the United States. Risks of potentially fatal complications of cardiac toxicity and death associated with prolonged terbutaline use led the FDA to issue a black-box warning in February 2011 stating that it should not be used for more than a 48–72 h period. These safety concerns, in addition to lack of superior efficacy compared to other tocolytics with preferable safety profiles, such as nifedipine, have limited the use of terbutaline in the United States in recent years.[654]

Labor Induction

Some maternal and fetal conditions may develop during pregnancy that increase the chance of adverse perinatal outcome if pregnancy continues. When delivery is felt to increase the likelihood of good perinatal outcome compared to allowing the pregnancy to progress, interventions to expedite the process of parturition may be indicated. When delivery is medically indicated, for either maternal or fetal benefit, in a pregnancy that has not begun the process of labor, artificial means to initiate labor are commonly employed. A variety of pharmacologic and nonpharmacologic interventions have proven efficacious to initiate parturition in pregnancies that have not begun the labor process. It has been estimated that over 20% of births in the United States result from induction of labor.[655]

Pharmacologic agents used for labor induction: PGs are often used for cervical ripening prior to labor induction when the cervix is unfavorable. A variety of different PG preparations have been studied, most with proven efficacy for yielding cervical ripeness. However, as a class, they have not been shown to decrease the frequency of failed induction or reduce cesarean delivery rates in nulliparous women.[655] Nevertheless, it is standard practice for women with an unfavorable cervix undergoing labor induction to receive either a mechanical or pharmacologic agent for cervical ripening prior to induction with oxytocin.

Intravaginal or intracervical PGE_2 (dinoprostone) or intravaginal PGE_1 (misoprostol) are commonly used cervical-ripening agents. Administration of PGE_1 tablets is the most common approach for cervical ripening given its low cost and ease of administration, in addition to some evidence of superior efficacy for achieving cervical ripening or delivery within 24 h compared to other agents.[656] Although PGE_1 has clinical and cost advantages compared to PGE_2 and OT, it is associated with more frequent complications related to uterine hyperstimulation (uterine tachsystole) in most studies performed to date.

Synthetic OT is the most frequently utilized medication for induction of labor. Uterine response to OT is very rapid, with uterine contractility demonstrated within 3–5 min of administration. The half-life of OT is 3 min, and discontinuation of infusion often results in decreased frequency of uterine contractility within a short period of time. Although frequently used on labor and delivery units globally, rare complications such as water intoxication and inadvertent overdose of OT have been reported with serious consequences. OT should be administered only by individuals adequately trained in proper dosing and in monitoring for adverse maternal and fetal effects.[655]

CONCLUSION

Pregnancy is a temporary state in the viviparous reproductive cycle that ends in a controlled manner and at a specific time in gestation (i.e., usually term) by the process of parturition. In general, the gravid uterus is highly proficient at expelling its contents, and as such dysfunctional parturition is mainly due to errors in timing rather than process. Major problems arise for the neonate when parturition occurs before term because it may be born before organ systems needed to maintain homeostasis outside of the uterus are mature. In contrast, a postterm fetus will be mature enough to survive as a neonate, but becomes too large to pass through the birth canal. In this case, parturition that is initiated postterm may not progress to delivery and instead puts the survival of both the fetus and the mother at risk. The timing of parturition is therefore critical for the success of pregnancy (and reproduction), and as such has been subjected to significant selective pressure through evolution. Consequently, considerable diversity exists among viviparous species in the normal length of gestation (i.e., term) and neonatal maturity at birth.

Examination of the comparative biology of birth timing[248] across various species demonstrates how natural selection has acted so that term for each species—and, by implication, the mechanisms that initiates parturition—matches a reproductive strategy to maximize overall reproductive efficiency. Thus, in addressing the subject of this chapter, the physiology of parturition was considered in a species-specific context. This approach is especially relevant for understanding human parturition because the mechanism for its timing was likely affected by a constellation of traits unique to the hominid lineage (e.g., encephalization and bipedalism), the most important of which is encephalization. We argue that the extant mechanism for initiating human birth represents natural selection in response to the benefits of encephalization, which increased fetal brain and head growth, balanced against the cost of reproductive failure (i.e., neonatal and maternal loss) due to the difficulty of delivering a large-headed fetus through the limited pelvic outlet and/or the nutrient demand of the large fetal brain exceeding maternal nutrient provision. This problem seems to have been solved by the selection of a trigger mechanism for hominid parturition that shortened the length of gestation so that the fetus is born at a time when it is capable of surviving ex utero but before its head becomes too large to pass through the pelvic outlet. The implication of this evolutionary paradigm is that the fidelity of animal models to mimic the physiology of human parturition, particularly the initiating mechanism, is poor.

Nonetheless, certain elements in the hormonal control of parturition appear to be conserved in most viviparous species. The general consensus is that the hormonal interactions that promote pregnancy maintenance and the process of labor and delivery generally involve a common group of uterotropic (progesterone and estrogens) and uterotonic (OT and PGs) hormones. It is also generally accepted that parturition is blocked by progesterone and triggered by any interruption of progesterone actions mediated by the nuclear PRs (i.e., progesterone withdrawal). A major conundrum, however, derives from the fact that human parturition occurs without a systemic progesterone withdrawal, and this continues to be a major criticism of the progesterone block hypothesis. An equally valid alternative hypothesis is that human parturition does not require progesterone withdrawal but instead is promoted by pro-labor stimuli that overcome the progesterone block. Nonetheless, clinical studies of nuclear PR antagonists show that PR-mediated progesterone signaling is essential for the maintenance of human pregnancy and that disruption of PR signaling alone is sufficient to induce the full parturition cascade. In addition, studies of nuclear PR isoform signaling in myometrial cells suggest that progesterone withdrawal is mediated by multiple redundant mechanisms that desensitize the gestational tissues (myometrium, cervix, decidua, and fetal membranes) to PR-mediated progesterone actions, a process referred to as functional progesterone withdrawal. A key issue for future research will be to identify the upstream hormonal interactions that induce functional progesterone withdrawal in each of the gestational tissues. These mechanisms will likely reflect the human-specific pathways that determine the timing of birth. Importantly, this understanding will likely reveal therapeutic strategies to maintain progesterone signaling in the various gestational tissues and prevent preterm birth.

A general theme that is emerging from research in multiple species is that the hormonal control of parturition involves positive-feedback loops at the paracrine level within each of the gestational tissues and at the endocrine level between the uterus and extrauterine sites, including the maternal hypothalamus (Figures 42.7 and 42.9). Tissue-level inflammation appears to be a major contributor in this regard, and myometrial and cervical distention may also be involved. CRH, glucocorticoids, OT, and PGs may all contribute to the positive-feedback acceleration of labor. This paradigm implies that the drive to empty the uterus is a reflexive response driven by positive-feedback interactions. Based on this model, the success of pregnancy depends on repression of the positive-feedback interactions until term. Future research should be targeted to unravel the dynamics and interconnectivity of the various positive-feedback loops in each of the gestational tissues, and between the uterus and the maternal pituitary, and determine the mechanisms by which those interactions

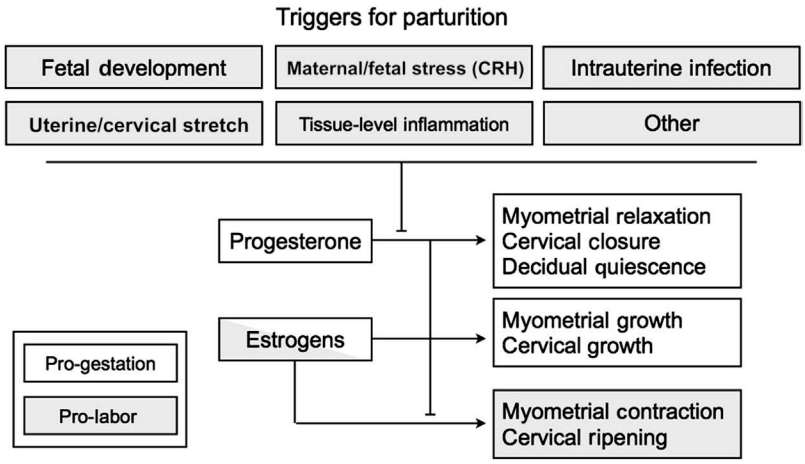

FIGURE 42.9 Schematic representation of the triggers for parturition. Uterine quiescence and growth are promoted by the combined actions of progesterone and estrogens. Estrogens also promote pro-labor gene expression at the time of parturition, but this activity is inhibited by progesterone for most of pregnancy. Multiple physiological triggers for parturition function primarily by inhibiting the pro-gestational actions of progesterone.

are controlled by progesterone and other hormones involved in pregnancy maintenance and the induction and process of parturition.

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CHAPTER

43

Maternal Adaptations to Pregnancy

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INTRODUCTION

A variety of reproductive strategies are found among vertebrates ranging from groups with relatively small maternal energy investment, as for some reptiles, to mammals that nurture offspring within the intrauterine environment and invest in costly postnatal care for extended periods of time. Mammals are also unique in their ability to nurture after birth in the form of milk from the maternal mammary gland that provides all nutritional requirements for a period of time. Lactation provides the opportunity for bonding and physical stimulation that is important in postnatal brain maturation.[1] This form of reproduction exacts a high energy cost from the mother in the form of remodeling of the maternal body, growing and maintaining augmented mammary tissue, carrying the weight of the developing fetus for the entire gestational period, and providing nutritional substrate in the form of milk to offspring as well as attending to the offspring for times ranging from weeks to months during the postnatal period.

This chapter will cover the current knowledge of major physiological changes that accompany pregnancy in women. Perhaps the most important adaptations of pregnancy are imperceptible even to a woman carrying a child, yet essential for a successful pregnancy. An inadequate adaptation to pregnancy can lead to improper fetal development or even fetal demise.

One must only review the state of knowledge a century ago to appreciate the amazing progress in our understanding of the physiology of pregnancy. In 1910 Marshall wrote, "What constitutes the original stimulus for the changes that occur in pregnancy remains still outside our ken." He further reported that "the blood pressure is not affected in normal pregnancy, but is always raised in labor as a result of uterine contractions".[2] At that time, the estrogenic sex steroid hormones had not been isolated and cardiovascular adaptations to pregnancy were little studied. But by the late 1960s a general understanding of the primary respiratory and cardiovascular changes in pregnancy were known and well described. For example, increases in blood volume and changes in respiratory function were known, as were basic aspects of metabolism in pregnancy and energy requirements. In 1968 Hytten coauthored a review of the known adaptations to pregnancy,[3] which were then detailed in a monograph.[4] Yet, in spite of decades of research on the mechanisms that underlie changes during pregnancy, the most fundamental cellular and molecular mechanisms remain beyond reach. Pregnancy-related changes in the cardiorespiratory and renal systems have been studied most and are, thus, best understood. This chapter will emphasize cardiovascular–respiratory–renal changes as examples of the elaborate modifications of the human body that make childbearing possible and will highlight crucial areas that need further investigation. Recent

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findings will be especially noted. Much of what we know about pregnancy has come from animal experimentation. However, the next decade should bring an avalanche of new data from human pregnancies as it becomes increasingly clear that population health is largely driven by the quality of growth in the womb and as noninvasive imaging methodologies become widely available.

Definitions

Fortunately, most of the terminology used in the pregnancy literature is not controversial. There has been a longstanding debate over the use of the term *adaptations* when applied to the pregnant condition because changes in body function during pregnancy are not adaptations as defined by evolutionists, such as a biological trait that confers biological advantage to an organism. Rather, in the case of pregnancy, adaptations are defined as those anatomic and functional changes in the female body that are believed to have physiological benefit to the growing embryo and fetus. This controversy has, over the decades, led authors to use other phrases, like "adjustments to pregnancy" or "physiological changes in pregnancy," both of which continue to be occasionally used. However, from the authors' view, there is no longer a need to apologize for this specialized use of *adaptation* since it has been firmly entrenched in the pregnancy literature for decades.

METABOLIC ADAPTATIONS

Energy Costs of Pregnancy

The total energy cost of a human pregnancy over its full-term duration has been estimated at some 80,000 kcal.[5,6] The average weight gain during pregnancy is about 12.5 kg and includes the increased weight of the fetus, amniotic fluid, uterine tissue, breast tissue, and blood, as well as extravascular and intracellular water. Changes in maternal body composition over the course of gestation have been studied.[7,8] Measuring the composition of body compartments during pregnancy is difficult, and no method has gained complete acceptance. Traditional methods like skin-fold thickness[9] are helpful but not well validated across populations. Newer methods like bioelectric impedance analysis, underwater weighing, air displacement plethysmography, and nonradioactive isotope dilution methods are likely to improve the accuracy of such measurements as they become standardized for pregnant women. Studies over the past few decades suggest that approximately 1 kg protein, 4 kg fat, and 7 kg water are gained during pregnancy.[4,10] Half of the protein weight accrues in the fetus and placenta. Pregnancy weight gain varies considerably among individuals, with very low or very high weight gains being related to poorer fetal outcomes compared to moderate weight gains.[11]

Thus, in 2009 the National Institute of Medicine updated guidelines for weight gain based upon the category in which a woman's prepregnancy body mass index (BMI) (weight per height squared, in kg/m^2) is found (Table 43.1). Less weight gain is recommended with each step increase in prepregnancy BMI.[8] Categories of recommended weight gain include: underweight, normal weight, overweight, and obese.

The basal metabolic rate (BMR) of the mother increases during pregnancy. It is the level of metabolism of an individual at rest, often determined by measuring oxygen consumption per minute. BMR increases from about 1300 kcal/day to about 1700 kcal/day over gestation in women of average prepregnant BMI.[11] Figure 43.1 shows the increases in BMR above prepregnant levels over gestation for three groups of women with different body mass indices. In reality, the total daily energy expenditure includes not only the BMR but also additional energy expended by daily activities (Figure 43.2). Total energy expenditure ranges from some 2700–3000 kcal/day at 36 weeks depending upon BMI at the time of conception and the degree of activity of the members in the three BMI groups.[11] Hytten and Leicht proposed that the increased energy costs of pregnancy could be met by a reduction of activity energy expenditure without an increase in food energy consumption.[4] While this proposal might be correct, it has not been tested. It is clear that most women expend excess energy from a high level of activity during pregnancy and require a significant increase in food intake.[11] There is evidence that metabolic rate is regulated differently in pregnant women compared to nonpregnant women. Before pregnancy, metabolic rate is most closely related to fat-free body mass whereas during the third trimester metabolic rate is most closely related to body fat mass.[16] This may mean that maternal metabolic rate is uniquely regulated by adipose reserves during pregnancy.[17]

Oxygen consumption ($\dot{V}O_2$) increases by some 50 ml/min in pregnant women by term.[18] Figure 43.3 shows the increases found in individual organs. As expected, the gravid uterus accounts for the largest increase in oxygen consumption. Term women sitting upright show increases in resting $\dot{V}O_2$ over nonpregnant women by some 33%, reflecting increased metabolism. There is evidence of a fundamental change in the regulation of the oxygen consumption threshold during pregnancy in sheep.[19] It is not known whether this occurs in humans. However, it is known that physiological conditions will affect oxygen consumption in pregnant women. For example, resting oxygen consumption is decreased by 15% in first trimester women who suffer from hyperemesis gravidarum, severe "morning sickness", and are unable to consume a normal daily calorie intake.[20]

TABLE 43.1 New Recommendations for Total and Rate of Weight Gain during Pregnancy, by Prepregnancy BMI[12]

	Total Weight Gain		Rates of Weight Gain[a] 2nd and 3rd Trimester	
Prepregnancy BMI	**Range in kg**	**Range in lbs**	**Mean (range) in kg/week**	**Mean (range) in lbs/week**
Underweight (<18.5 kg/m^2)	12.5–18	28–40	0.51 (0.44–0.58)	1 (1–1.3)
Normal weight (18.5–24.9 kg/m^2)	11.5–16	25–35	0.42 (0.35–0.50)	1 (0.8–1)
Overweight (25.0–29.9 kg/m^2)	7–11.5	15–25	0.28 (0.23–0.33)	0.6 (0.5–0.7)
Obese (≥30.0 kg/m^2)	5–9	11–20	0.22 (0.17–0.27)	0.5 (0.4–0.6)

[a]*Calculations assume a 0.5–2 kg (1.1–4.4 lbs) weight gain in the first trimester.*[13–15]

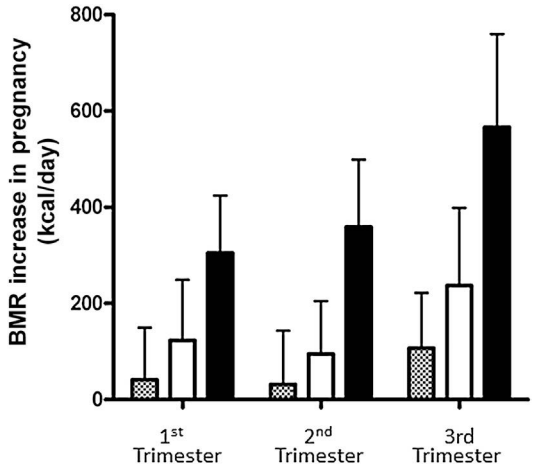

FIGURE 43.1 Changes in basal metabolic rate (BMR) during pregnancy at three time points, 9 weeks, 22 weeks, and 36 weeks, in three groups of women. The first group with a below-average body mass index before conception (BMI < 19.8 kg/m^2, hatched bar); the second group, a normal BMI (19.8–26.0 kg/m^2, unfilled bar); the third group, a high BMI (>26.0 kg/m^2, filled bar). *Source: Data from Ref. 11.*

Diet, Maternal Body Composition, and Fetal Programming

There is increasing evidence from animal[21–23] and human[24] studies that maternal diet affects the growth patterns of the placenta, fetus, and offspring. It is also clear that fetuses that experience growth faltering before birth are at risk for chronic diseases as adults.[25] Thus, evidence for links between maternal diet, fetal growth, and adult disease is becoming solidified. Shiell and coworkers studied the blood pressures of 626 young men and women of Motherwell, Scotland, whose mothers were recommended to eat a high meat, low carbohydrate diet during pregnancy.[26] The offspring of mothers who had the highest consumption of meat and fish had the highest mean systolic pressures. These findings point to the importance of maternal diet and body composition in establishing the health of their offspring in later life. The nutritional flow that is made available to a fetus depends on many aspects of the mother's nutrition and physiology, including her nutritional stores in the form of fat and muscle mass, her diet, and her metabolism, which is the product of her lifetime's nutrition.[27] Little is known about the fascinating increased drive for specific foods reported by many women during pregnancy.

Maternal appetite increases during pregnancy in a sex-specific way. Women carrying boys consume more calories from all macronutrient sources, protein, carbohydrate, and lipid.[28] Boys grow faster than girls with less relative placental mass and thus have placentas that are more efficient. The growth of boys is more closely linked to maternal body composition than is the growth of girls.[29] Because of the robust growth strategy of male fetuses, they are more vulnerable to nutrient deprivation.[30,31]

The powerful influence of the size and shape of the placenta on offspring risk for disease in later life has become apparent in recent years. Studies from Finland,[32] the United Kingdom,[33] the Netherlands,[31,33] Saudi Arabia,[34] India,[35] and the United States[36] have brought new emphasis to the importance of placental growth patterns as predictors of disease. Figure 43.4 shows placental phenotypes that are related to offspring disease.[37]

Not only is placental size and shape important but also the combination of placental and maternal phenotype may have even greater predictive value. These relationships suggest the presence of biological processes by which maternal body composition interacts with fetal/placental development leading to constraints on fetal organ development. Placental morphological studies have shown that placental breadth, length, thickness, and the better-known effects of placental weight are each associated with specific chronic diseases in the adult.

CARDIOVASCULAR ADAPTATIONS

Blood Volume

Maternal blood volume increases over time beginning at 6–8 weeks gestation, reaching a maximum at approximately 32–34 weeks, and changing little thereafter.[38–42] Maternal blood volume expansion supports fetal growth. Pregnancies complicated by fetal intrauterine growth restriction have lower mean maternal plasma

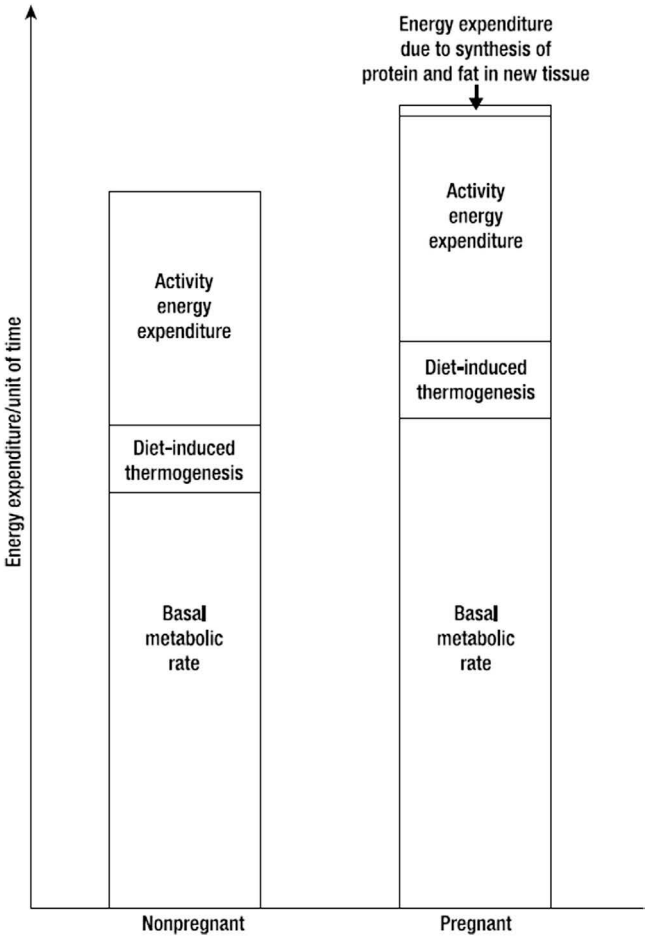

FIGURE 43.2 Increases in energy expenditure during pregnancy. *Source: Data from Forsum E, Lof M. Energy metabolism during human pregnancy.* Annu Rev Nutr *2007;***27***:277–292.*

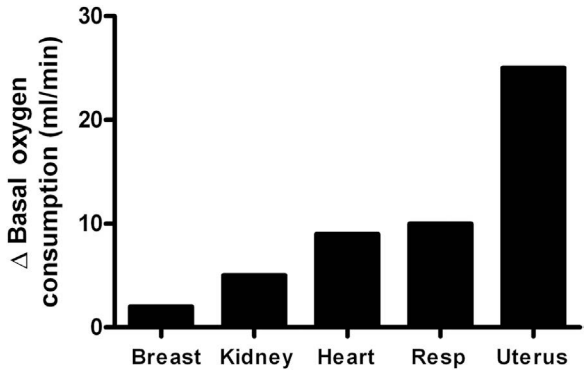

FIGURE 43.3 Increases in basal oxygen consumption (ml/min) for individual organs in near-term pregnancy compared to nonpregnant consumption rates. *Source: Data from Ref. 18.*

volumes than do pregnancies with normal fetuses (2976 ± 76 ml versus 3594 ± 103 ml, respectively).[31] The increase in blood volume serves at least two purposes: (1) it facilitates maternal and fetal exchanges of respiratory gases, nutrients, and metabolites, and (2) it reduces the impact of maternal blood loss at delivery. Blood losses of 300–500 ml for vaginal births and 750–1000 ml for Caesarean sections are typical and are compensated to some degree by the so-called "autotransfusion" of blood from the contracting uterus. Further blood losses tap the resources of maternal stores.

Bernstein and associates detected plasma volume expansion in pregnancy during the sixth week following a woman's last menstrual period.[43] By 12 weeks, plasma volume had expanded by approximately 14 ± 12% (mean ± standard deviation, SD) over follicular phase estimates. While the average blood volume increases from a prepregnant 6.5 l to 8.5 l during pregnancy, increases vary considerably among women, ranging from 30% to 50% in proportion to the number of fetuses[44,45]; increases have been estimated in a singleton pregnancy at 1570 ml and 1960 ml in a twin pregnancy.[44] Women show a similar plasma volume expansion pattern in their subsequent pregnancies.[40,44] Most of the added volume of blood is found in the uterus, breast, kidney, striated muscle, and skin.

The increase in plasma volume during pregnancy is relatively greater than that of red cell mass (20–30%) resulting in a decrease in hemoglobin concentration. Figure 43.5 shows that the decrease in hemoglobin concentration is not the result of simple hemodilution; both red cell mass and blood volume are increased. Thus, the popular term *hemodilution of pregnancy* is an oversimplification. Supplemental iron and folic acid intakes are necessary to restore hemoglobin levels to normal pregnant levels (~12 g/dl) if a woman has been iron deficient. Leukocyte counts are variable during gestation but remain at the upper limits of normal; leukocyte production appears to be regulated independently of red cell production. Like red cell concentration, plasma protein concentrations and total plasma osmolality decrease during pregnancy.

The mechanism by which blood volume increases is not fully understood, yet there are a number of physiological changes that are thought to be important. The role of the kidney is discussed in detail following. There is evidence that systemic vascular resistance is decreased under the influence of gestational hormones, which leads to activation of the renin–angiotensin–aldosterone system causing increases in sodium retention and blood volume.[46–48] There is evidence from experiments in baboons that systemic vasodilation precedes the measured increase in maternal blood volume as pregnancy progresses.[49]

The placenta may also contribute to the hormonal milieu that affects maternal blood volume regulation. Placental estrogen synthesis is dependent upon the estrogen precursor (dehydroepiandrosterone) from the fetal adrenal gland. Estrogen stimulates the renin–angiotensin system and thus promotes aldosterone production. Plasma

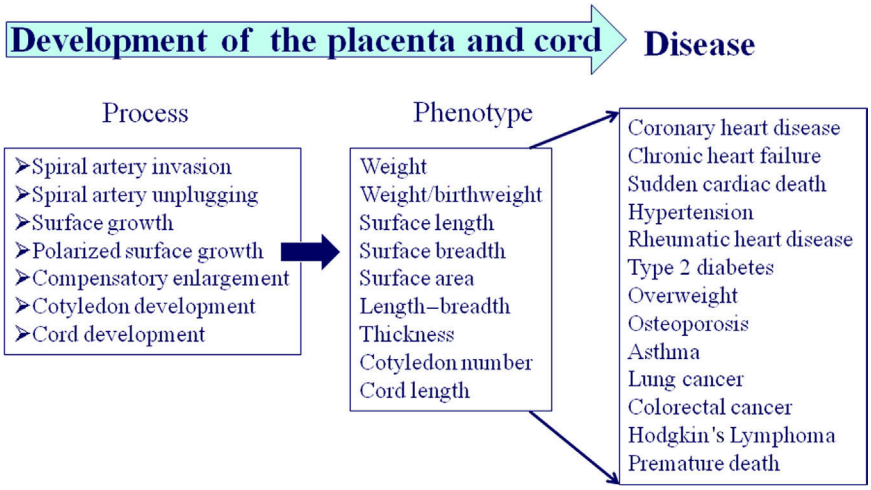

FIGURE 43.4 Placental phenotypes related to offspring disease. *Source: Data from Ref. 37.*

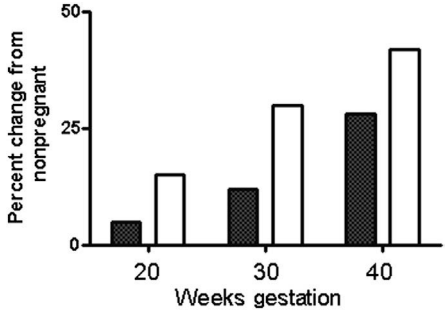

FIGURE 43.5 Average red cell mass (filled bar) and blood volume (open bar) increase over the course of pregnancy. *Source: Data from Ref. 41.*

renin levels, which are high during pregnancy, ensure high levels of angiotensin II (Ang II) that stimulate arginine vasopressin (AVP) release and water retention.[46,48,50,51] In the kidney, increased mineralocorticoid activity may also be important in the regulation of renal function during pregnancy.[52] Extra-adrenal conversion of progesterone to deoxycorticosterone also increases mineralocorticoid activity.[53] There is a net accumulation of sodium during pregnancy of some 500–900 mEq.[54,55] Further studies are needed to integrate these physiological features of pregnancy. (There are a number of interesting features of the physiological adaptations to pregnancy.)

Altitude

The hypobaric hypoxia of altitude causes an increase in hematocrit and hyperviscosity in long-term residents. Pregnant women adjust to altitude (~4400 M) differently from women at sea level in that they show blunted adaptive hematological adaptations in general.[56] Hematocrit and viscosity decrease with gestation in women at altitude but much less than for women at sea level. Thus, women at altitude have a higher blood viscosity than women at sea level even during pregnancy.

Circulating Factors with Cardiovascular Effects

A number of circulating factors are important in blood volume regulation. Many of these are also powerful regulators of vascular tone and of vessel remodeling. Atrial natriuretic peptide (ANP) is maintained at normal levels during pregnancy or is increased depending upon posture.[57,58] Figure 43.6 shows plasma ANP levels from 15 healthy women as a function of gestational age and posture.[59] In healthy men and nonpregnant women, plasma osmolality, plasma renin activity, and plasma concentrations of AVP, ANP, and aldosterone all increase with increasing exercise intensity. However, in nonpregnant individuals, high intensity exercise has an antidiuretic and antinatriuretic effect, opposite to the effects expected when plasma ANP levels are elevated.[60] Heenan and colleagues studied the responses to exercise in pregnant women, expecting that their hypervolemic state would attenuate the normal increases in Ang II and AVP.[61] Thirteen pregnant women of 37 weeks gestation were studied at rest and at two intensity levels as a percentage of the estimated anaerobic threshold (VT value). Figure 43.7 shows that plasma Ang II levels were high in these pregnant women but tended to decrease with increasing exercise intensity; in contrast, nonpregnant women had lower initial Ang II levels that increased with exercise. The AVP response to exercise was completely blunted in the pregnant women. However, plasma levels of ANP were elevated with exercise.

Verkeste and associates found that the increase in plasma volume during pregnancy in rats results from fluid retention rather than from a shift within the extracellular fluid volume (ECFV) from the interstitium to the intravascular compartment.[62] They found a gradual synchronous increase in all fluid compartments, without consistent change in their relative distributions. Chapman and associates found that peripheral vasodilation occurs in the luteal phase of the normal menstrual cycle in association with an increase in renal plasma flow and filtration.[63] They found increased activation

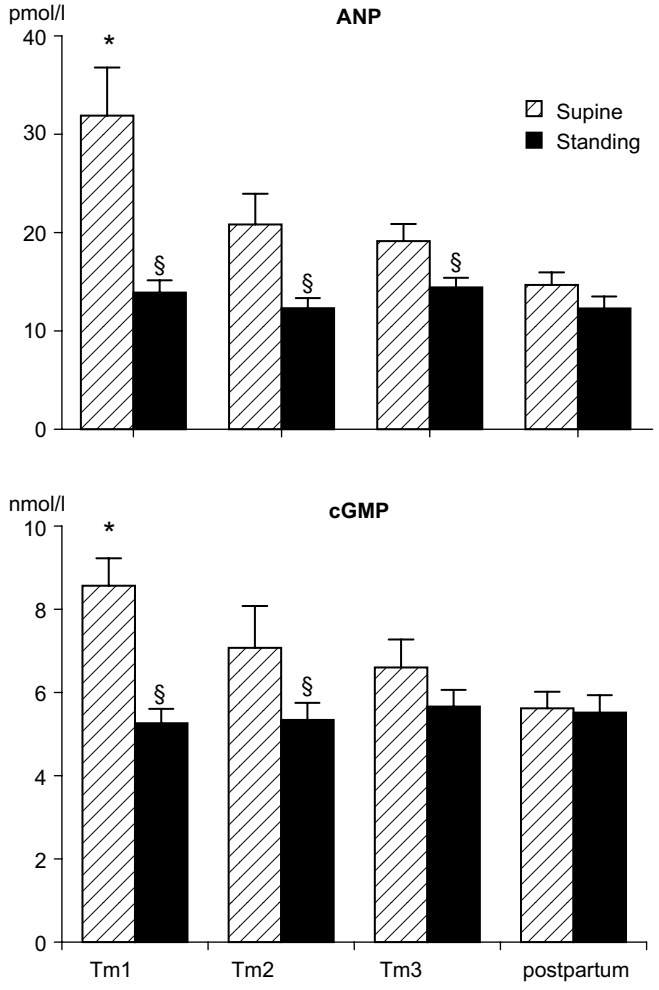

FIGURE 43.6 **Plasma atrial natriuretic peptide (ANP) and cGMP at each trimester (Tm) of pregnancy and postpartum in 15 healthy women in the supine and upright positions (mean ± SEM).** *$P < 0.05$ versus postpartum in the same position; §$P < 0.05$ versus corresponding supine value. *Source: From Ref. 59, with permission.*

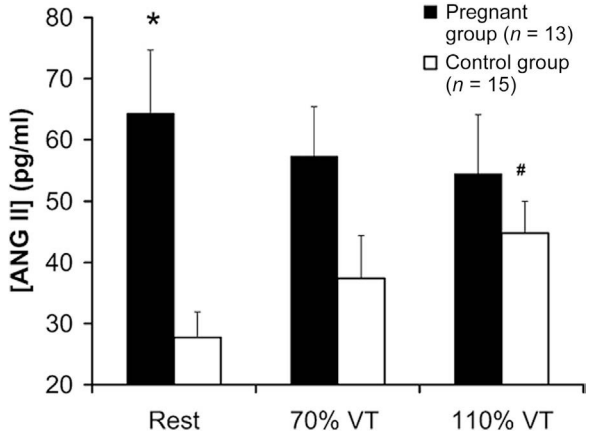

FIGURE 43.7 **Plasma angiotensin II concentration ([ANG II]) at rest and at two work rates as a percentage of the estimated anaerobic threshold or ventilatory threshold (VT).** *Significant difference ($P < 0.05$) between groups. #Significant change ($P < 0.05$) within group from rest. *Source: From Ref. 61, with permission.*

of the renin–angiotensin–aldosterone system indicating that vasodilating mediators may be responsible for the observed hemodynamic changes. The authors suggest that hormonal changes during the normal menstrual cycle mimic changes seen in early pregnancy. This would argue for changes in hormonal environment as the primary driving force for changes in blood volume regulation.

Atrial Distension

The role of atrial distension in regulating maternal blood volume has been investigated and is controversial. Some scientists have suggested that the maternal atrium grows to accommodate increased blood volume, which, in turn, decreases the degree of stretch and blunts the stretch-ANP release response. That explanation is not likely to be true for two reasons: (1) an increase in systemic blood volume would not necessarily increase atrial transmural pressure, and (2) an increase in the radius of curvature of the atrial wall would increase wall stress and presumably make the atrium more sensitive to ANP release unless there was a disproportionate increase in wall thickness. Thus far, no study has provided convincing evidence for an increase in atrial stretch during pregnancy. Kaufman used a rat model to study whether pregnancy affects the sensitivity of atrial distension in the release of ANP and whether neural input from the atrial volume receptor is altered.[64] Atrial distension was achieved by implanting small balloons positioned at the superior vena caval/right atrial junction of rats; inflation of the balloon did not impede blood flow through the heart. In virgin rats, atrial stretch caused increased urine production, urine sodium and potassium output, and decreased free water clearance; plasma ANP levels also increased. No such response was observed in pregnant animals. Distension of isolated atria derived from unmated and 7-day pregnant rats resulted in an increase in secretion of ANP into the perfusate, whereas atria from 14- to 21-day pregnant rats were unresponsive to distension. The authors concluded that there is attenuation of both hormonal and neural responses to atrial distension in the pregnant rat. The sensitivity of ANP release from the heart may accommodate blood volume increases in pregnant women without eliciting homeostatic mechanisms to eliminate the extra fluid.

Deng and Kaufman found that atrial distension caused an increase in c-fos expression in the paraventricular nucleus of the lateral hypothalamus in rats.[65] During pregnancy, this response was markedly attenuated. Zhang and Kaufman sought to determine whether nitric oxide (NO) was involved in cardiovascular homeostasis in pregnancy.[66] They found that nitric oxide synthase (NOS) inhibition selectively reduced plasma volume in pregnant rats. Lo and Kaufman found that plasma levels of 5 alpha-pregnan-3 alpha-ol-20-one (pregnan), a potential regulator of NOS, is increased during pregnancy in rats.[67] One

milligram of pregnan given to rats over a two-day period stimulated NO biosynthesis and an increase in plasma volume in nonpregnant rats. These data are interesting in that while NO is known to be a powerful vasodilator in pregnancy, it may also affect cardiac function.[68]

During pregnancy in most species, the resting levels of plasma ACTH (corticotropin), aldosterone, cortisol, and Ang II are increased.[69,70] Cortisol is undoubtedly important in blood volume regulation. For example, when maternal cortisol is decreased experimentally, blood volume during pregnancy is reduced in sheep.[71]

Lindheimer and Davidson have shown that the osmotic thresholds for thirst and antidiuretic hormone release simultaneously decrease in pregnancy so that water intake is increased, retained, and body fluids are diluted.[50] In pregnancy, AVP release is not suppressed at the usual level of body tonicity so that ingested water is retained. With increased body water, plasma osmolality declines until it is beneath the osmotic thirst threshold, and a new steady state is established. Pregnancy is characterized by increments in intravascular volume, but volume-sensing mechanisms appear to accommodate as gestation progresses so that each new volume increment is "sensed" as normal. The metabolic clearance of AVP increases several fold, due to rising plasma cystine aminopeptidase (vasopressinase), which is produced by the placenta.

Vasopressin and ACTH responses to hypotension are altered in pregnant ewes,[69,72,73] and the relationship between mean arterial pressure and vasopressin or ACTH response shifts, consistent with a change in set point for regulation of mean arterial pressure. The decrease in hormone responses to hypotension is stimulus specific; ACTH responses to hypoglycemia are increased in the pregnant ewe and AVP responses to hyperosmolality are not altered in the pregnant ewe.[69,72]

The heart rate responses to hypotension are also decreased in pregnant ewes, consistent with the observations in the rat that baroreflex responses are decreased in the pregnancy. Recent studies suggest that the decrease in baroreceptor gain in pregnancy is related to decreases in central insulin concentrations in rabbit and rat.[74] The vasopressin and cortisol responses to hypotensive hemorrhage are also altered in the pregnant dog[75,76]; the relation between mean arterial pressure and hormone concentration is depressed. These data indicate that pregnant animals have a reduced ability to maintain arterial pressure during hemorrhage compared to nonpregnant animals. In addition, increases in heart rate, renal sympathetic activity, vasopressin, ACTH, and cortisol that are mediated by the baroreceptor reflex are reduced during pregnancy.[76]

Circulatory Adaptations

Remodeling of the entire maternal circulation begins within a few weeks following implantation. Circulatory changes continue through parturition, after which most of the changes are reversed. Table 43.2 summarizes many of the changes that occur. Among the most profound changes are the early decreases in systemic vascular resistance and increases in cardiac output. These changes occur even before there is an increase in metabolic rate.[77] The heart is remodeled with increased left ventricular chamber dimensions and increased left ventricular mass, reminiscent of changes observed in athletes.[78–81]

Cardiac Output

Cardiac output increases by more than 50% during pregnancy (Figure 43.8). It peaks by mid-third trimester, but half of that increase takes place within the first 8 weeks, long before $\dot{V}O_2$ has increased. Thus, the increase in cardiac output is not the sole result of increased tissue demand.

$$\dot{Q}_{CO} = SV \times HR$$

Because cardiac output is the product of stroke volume and heart rate, each has the potential to augment cardiac output in pregnancy; Figure 43.8 shows that both are important. Heart rate and stroke volume increase early in gestation and both reach their peak at mid-gestation. Stroke volume reaches a plateau by about 20 weeks and appears to gradually decrease over the remaining course of gestation. The magnitudes of change in stroke volume and heart rate are quite variable between individuals, and some studies have shown somewhat different patterns for these changes.

For any given chamber volume, stroke volume is regulated through increases in end diastolic pressure, which changes cardiac chamber wall stress (preload, the force/area in the myocardium), by decreases in systolic load (afterload, the force/area acting on the muscle wall during ejection), and by contractility (the contractile state within the myocardium). Of these three, only the decrease in afterload is a well-accepted mechanism for increasing stroke volume during pregnancy. While there are many investigators who have found increases in

TABLE 43.2 Hemodynamic Changes in Pregnancy[17]

Increased	Decreased
Blood volume	Systemic vascular resistance
Uterine blood flow	Pulmonary vascular resistance
Red cell mass	Hematocrit
LV diastolic dimension	Arterial blood pressure
Stroke volume	Plasma albumin concentration
Heart rate	Arterial carbon dioxide tension
Arterial oxygen tension	Arterial hydrogen ion concentration
Arterial compliance	Colloid osmotic pressure

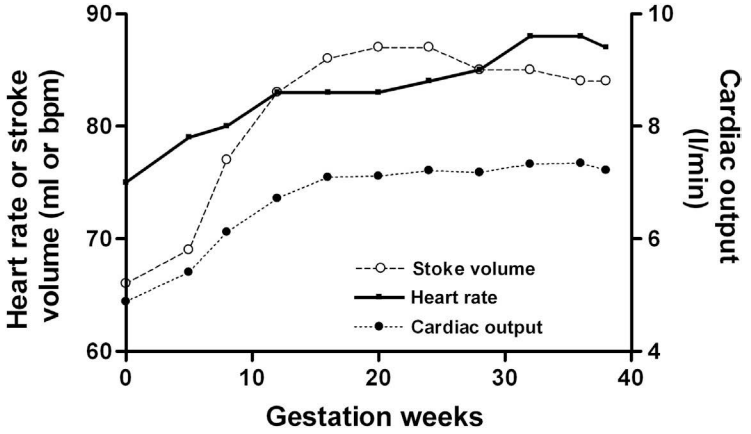

FIGURE 43.8 Cardiac output (closed circles) increases over gestation as the product of increased stroke volume (open circles) and heart rate (solid line). *Source: Data from Ref. 80. Reproduced from Ref. 17, with permission.*

surrogate measures for preload, an equal number have not seen increases. However, the heart of the pregnant woman may be quite sensitive to changes in preload so that profound alterations in cardiac anatomy could be driven by preload changes that are hardly measurable. One important factor that must be considered when measuring central venous or end diastolic pressure is maternal posture. As for all adults, preload increases in the supine position compared to sitting upright. However, in the later stages of pregnancy, the weight of the uterus may compress the inferior vena cava in the supine posture and can change filling pressures of the heart in a dramatic way. The importance of posture in determining plasma ANP levels is shown in Figure 43.6.

There is an even greater controversy over the role of contractility as an adaptation to pregnancy. Some authors have found increases,[82] others have agreed with the Poppas study[83] that there is no change,[84] and yet others have found a decrease in contractility.[85,86] Differences in contractile state are notoriously difficult to measure in humans using noninvasive methods in which estimates are affected by the loading conditions of the heart. In the Poppas and Mone studies, contractility was estimated from a method believed to be load and heart rate independent, the velocity of circumferential fiber shortening relative to the stress in the wall at end systole. Yet the findings of these two groups remain different. Thus, the degree to which preload and contractility are changed during pregnancy requires further study.

Cardiac output also increases during labor. Increases may reach 30% in the first stage and more in later stages, with variability dependent on posture and anesthesia.[87] Cardiac output increases are more related to stroke volume, through increases in venous filling than to increases in heart rate.[88]

Arterial Blood Pressure

Mean arterial blood pressure decreases over the course of pregnancy and is lowest at mid-gestation. Systolic and diastolic pressures, as well as pulse pressure, drop modestly during pregnancy and reach their lowest values at about 20 weeks. This drop in pressure is the result of decreased systemic vascular resistance (Figure 43.9). The behavior of the cardiovascular system can be understood in simplified form by applying a form of Ohm's law for direct current in a simple circuit. Under such conditions, current I is equal to the ratio of applied voltage divided by total resistance. In equation form:

$$I = \Delta V / R.$$

Following the same form for the systemic circuit of the cardiovascular system, the total flow through the system, cardiac output ($\dot{Q}_{CO}$) is equal to the driving pressure across the systemic circuit, aortic pressure minus right atrial pressure ($P_{ao} - P_{ra}$) divided by the total vascular resistance of the circuit (TVR). In equation form:

$$\dot{Q}_{CO} = (P_{ao} - P_{ra}) / \text{TVR}$$

Systemic arterial pressure decreases during the first trimester of pregnancy. Systolic, diastolic, and mean arterial pressure decrease about 10% by the seventh week of pregnancy.[42] Because, in an oversimplified view, arterial pressure is determined by the product of cardiac output and TVR, one or both of these factors must decrease to explain a decrease in arterial pressure.

$$\text{BP} = \dot{Q}_{CO} \times \text{TVR}$$

Because cardiac output is increased early in pregnancy, it cannot account for a decreased arterial pressure in pregnancy. However, TVR is the change that leads to decreased blood pressure. Thus, total resistance to flow must be decreased to a greater degree than cardiac output is increased, leading to a drop in arterial pressure. It has been argued that the reduction in TVR is due to the presence of the dilated uterine bed. However, the contribution of the gravid uterus, at its peak in flow, has been estimated to contribute

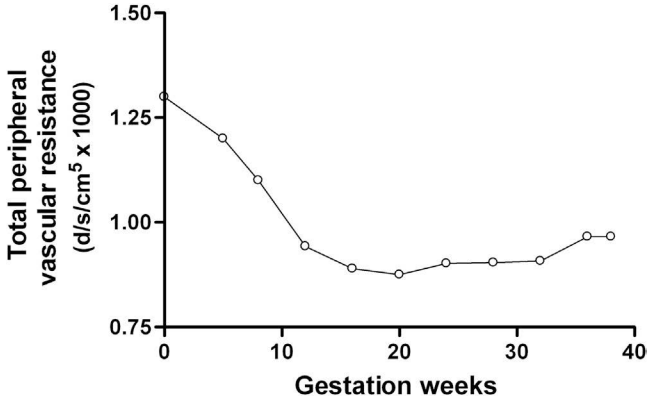

FIGURE 43.9 **Total peripheral resistance decreases dramatically over the first 16 weeks of pregnancy.** *Source: Data from Ref. 80. Reproduced from Ref. 17, with permission.*

about 20% to the decrease in TVR. Both systolic and diastolic blood pressure decrease until about 28 weeks and then gradually approach prepregnant values by term.[46] Blood pressure values are sensitive to the methods used to make the measurement. Ginsberg and Duncan found that mean systolic and diastolic blood pressures were lower by 6 and 15 mmHg, respectively, when determined by catheter in the radial artery compared to sphygmomanometry.[89] Similarly, Kirshon and associates found a lower systolic blood pressure by automated sphygmomanometry compared with a direct radial arterial measurement, but diastolic pressure was not different.[90]

It is well known that hypertensive disorders are a leading cause of maternal and perinatal morbidity and loss of life.[91] It has been estimated that some 25% of cases of intrauterine growth retardation are associated with inadequate cardiovascular adaptations to pregnancy and especially insufficient remodeling of the uterine arterial vascular bed.[92] There are a number of known causes of hypertension in pregnancy, and there are undoubtedly causes that remain undiscovered. Steer and coworkers have shown that both hypertension and hypotension during pregnancy are associated with babies who are undergrown and who suffer high mortality.[93]

Among the adaptations to pregnancy are a reduced pressor response to vasoconstrictors such as Ang II, AVP, norepinephrine, and phenylephrine. A number of studies in whole animals and isolated vessels have sought to determine the underlying mechanisms for this adaptation.[94–99] D'Angelo and Osol found an increased sensitivity to vasoconstrictors within uterine arcuate arteries in late-pregnant rats compared to nonpregnant rats.[99] Clearly, more investigation is required for clarification of the sensitivities of resistance vessels to constrictor-dilator substances in the pregnant woman.

Changes in central venous pressures during pregnancy have been difficult to prove, but lower body venous pressures are known to rise under the influence of mechanical factors. From mid-pregnancy, the enlarged uterus compresses both the inferior vena cava and the lower aorta in the supine position. Obstruction of the inferior vena cava reduces venous return to the heart leading to a fall in cardiac output by as much as 24% toward term when a woman is lying in the supine position. Most women compensate for the resultant decrease in stroke volume by increasing systemic vascular resistance and heart rate. Obstruction of the lower aorta and its branches can cause diminished blood flow to kidneys, uteroplacental unit, and lower extremities. During the last trimester, maternal kidney function is often lower in the supine than in the lateral position. Rarely, obstruction of the lower aorta and its branches can cause diminished blood flow to the placenta, which can result in insufficient transplacental gas exchange.[18]

Vascular Changes

Changes in vascular behavior with pregnancy have been studied in a number of animal models and in humans. Most studies show that arterial distensibility is increased over the duration of pregnancy. Whether veins behave in the same way is more controversial. The early literature suggests that venous compliance is increased under the influence of sex steroid hormones and that venous pressures in the lower limbs are elevated during pregnancy and prone to stasis.[100] Edouard and coworkers found that the distensibility and viscoelastic components of the lower limb veins decreased with duration in pregnancy and that upper limb veins made no such changes.[101] Vasodilator substances that are known to be important in pregnancy include estrogen,[102] NO,[103] prostacyclin,[102] relaxin,[104] and calcitonin-gene-related peptide.[105] These vasodilators have effects in a number of vascular beds including cerebral,[106] mesenteric,[105] coronary,[107] and uterine vessels.[102]

In sheep, vasodilatory prostacyclin and associated enzymes for its production are increased in the uterine arteries. Rupnow showed that estrogen and progesterone have independent effects on the production of prostacyclin-regulating enzymes in endothelium and vascular smooth muscle of uterine, renal, coronary, and omental vessels.[102] However, the combination of increased estrogen and progesterone, as seen with pregnancy, causes an increase in phospholipase 2 and cyclooxygenase in the uterine artery. Thus, changes in vascular remodeling and behavior are vessel specific.

The mechanisms that underlie the remodeling of the uterine vascular bed are the subject of intense investigation because the stakes in terms of fetal health are so profound. It is now well recognized that the uterine arteries of pregnant animals are extensively remodeled, being profoundly dilated in pregnancy compared to the nonpregnant state.[97,103,108]

Twin Pregnancies

Maternal cardiovascular responses to pregnancy are exaggerated in twin pregnancies compared to singletons. Twin pregnancies carry a higher risk for gestational hypertension and preeclampsia.[109,110] Kametas and coworkers reported that cardiac output is 20% greater, stroke volume is 15% greater, and heart rate is slightly higher in twin pregnancies than in singleton pregnancies.[111] Left atrial diameter and left ventricular end diastolic dimension are also larger. Others have found similar increases in cardiovascular hemodyamics.[79,82,109,110]

Pulsatile Characteristics of the Cardiovascular System in Pregnancy

As mentioned previously, the use of Ohm's law to characterize the circulation, as one would an electrical circuit, is the most straightforward way to understand the relationships between steady flow, driving pressure, and resistance to flow. Using these relationships, we are able to understand changes that occur in the cardiovascular system under steady load conditions during pregnancy. However, because an intermittent pump drives blood flow, many of the characteristics of the circulatory system can be understood only by analyzing the relationships of pulsatile pressure and flow.

Poppas and coworkers have assessed both steady and oscillatory aspects of the cardiovascular system using noninvasive measurements in 14 pregnant women during each trimester of pregnancy and postpartum.[83] They used instantaneous subclavian artery pulse tracings, Doppler flow velocities, and left ventricular M-mode echocardiography (Figure 43.10). Their measurements of steady hemodynamics and cardiac anatomy fit with previous reports. They found a pregnancy-related increase in left ventricular muscle mass and end diastolic chamber dimension. They did not detect changes in myocardial contractility.

Poppas and coworkers found that global compliance in the subclavian artery increased with gestational age as TVR decreased.[83] Global arterial compliance is the relationship between volume and pressure change during exponential diastolic pressure decay in an arterial segment ($\Delta V/\Delta P$).[112,113] This finding is undoubtedly related to well-documented changes in distensibility of arteries and veins during pregnancy under the influence of estrogen and relaxin as discussed earlier.[101,114–117] There are two mechanisms that could alter the distensibility of the arterial tree at a given transmural pressure: (1) the vascular wall might be remodeled so that matrix elements have an altered behavior, and (2) vascular smooth muscle tone might be reduced. Unfortunately, simply testing the pressure volume characteristics of specific vessels does not adequately distinguish between these possibilities under in vivo conditions.

One of the most important contributions of the Poppas study was the finding that oscillatory power (time related work, $\dot{W}$) was progressively increased in pregnancy compared to the nonpregnant state (Figure 43.11). This suggests that an ever increasing fraction of the energy expended by the muscle pump to circulate blood through the vascular tree is spent on overcoming the oscillating resistance offered by its vascular elements. Thus, as pregnancy proceeds, the circulation of blood requires extra energy by the heart, which has adapted to the pregnant condition.

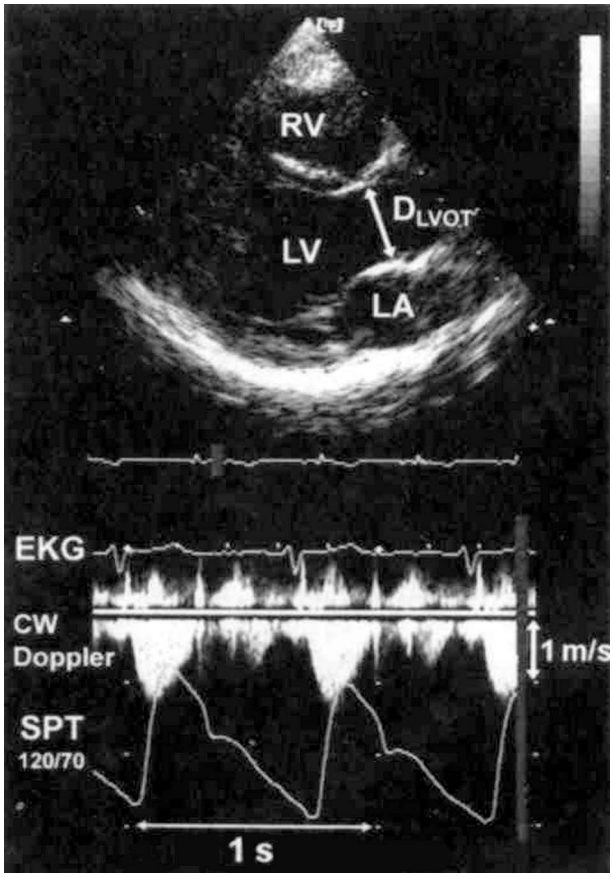

FIGURE 43.10 Two-dimensional parasternal long axis echocardiographic view showing measurements of left ventricular outflow tract diameter (LVOT). Simultaneous EKG, flow velocity (CW Doppler) and pressure profile from subclavian pulse tracing (SPT) in pregnant woman. *Source: From Ref. 79, with permission.*

Hematological Factors

Blood volume gradually increases by an average of some 50% by term but with great individual variability.[40] Pritchard reported an average increase in red blood cell mass of around 30% (450 ml) in both singleton and twin pregnancies as determined by the chromium (^{51}Cr)-labeled red blood cell technique.[44] Thus, it has been known for decades that plasma erythropoietin levels and consequent erythropoiesis increase during pregnancy.[118] However, recent studies have shown that red cell mass is suppressed in the first trimester as is erythropoiesis.[119,120] Figure 43.12 shows that hematocrit decreases with gestational age even as erythropoietin increases. The "set point" for

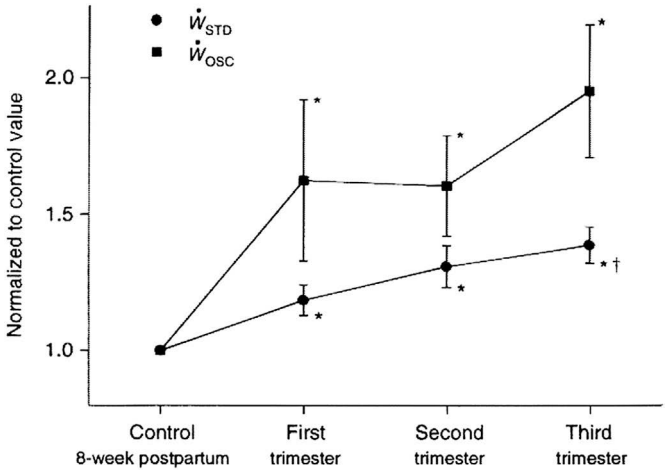

FIGURE 43.11 Increases in steady ($\dot{W}_{STD}$) and oscillatory ($\dot{W}_{OSC}$) power during gestation. Data are normalized to 8-week postpartum control values (mean ± SEM $^{*}p < 0.05$ versus control, $^{\dagger}p < 0.05$ versus first trimester). *Source: From Ref. 83, with permission.*

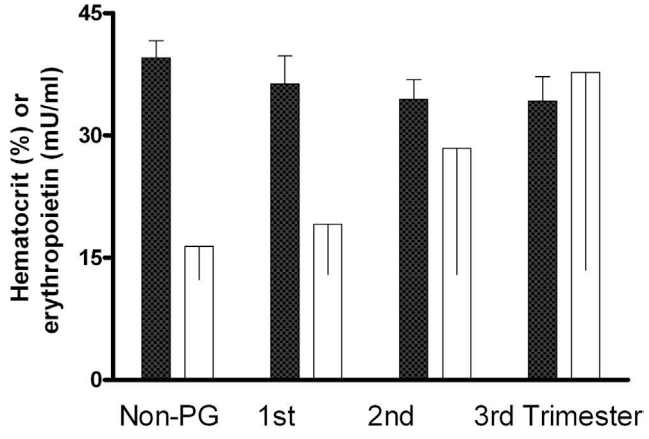

FIGURE 43.12 Hematocrit (filled bars ± SD) and plasma erythropoietin concentrations (unfilled bars) in nonpregnant women and in pregnant women in each trimester. *Source: Data from Ref. 119.*

red cell production appears to be altered with pregnancy because the rate of erythropoiesis is never adequate to maintain hematocrit at prepregnant levels. Thus, the increase in red cell mass during pregnancy is less than the increase in plasma volume and leads to the so-called "anemia of pregnancy".[40,121] It has been suggested that the availability of iron may limit erythropoiesis. Iron stores may be suboptimal in up to two-thirds of reproductive-age women before pregnancy because of recurrent iron loss from menstruation.[122] However, virtually all women have depressed hematocrits during pregnancy, regardless of iron stores.[119] Therefore, the iron limitation hypothesis is unlikely to explain the decrease. It is important to emphasize that the decreased hematocrit is not by simple hemodilution as is often stated in textbooks. As mentioned, red cell production rate increases during pregnancy, but increases less than required to maintain red cell concentration in the face of increased plasma volume. Like red cell concentration, plasma protein concentrations, and total plasma osmolality also decrease during pregnancy, which decreases blood viscosity.

Hemostasis

There is a commonly held view that maternal blood clots more easily during pregnancy. Some have argued from a teleological view that this hypercoagulability is related to the need to prevent excess hemorrhage with delivery of the placenta. However, a more important factor in stemming hemorrhage at birth is contraction of uterine smooth muscle, which occludes blood vessels in the uterus as the placenta is delivered. Regardless, the alteration in hemostasis with pregnancy carries an increased risk of thromboembolism. Thus, pregnant women are thought to be at increased risk for fatal pulmonary embolism and coronary occlusive disease.[123,124] The hypercoagulability is related primarily to changes in coagulant and anticoagulant proteins in the blood.[125] Table 43.3 shows coagulation proteins that are increased or decreased with pregnancy. Of note, procoagulant activity in the blood is primarily due to increases in factors VII, VIII, X, as well as fibrinogen and von Willebrand factor. Factor VII increases dramatically, up to 10-fold, whereas plasma levels of fibrinogen may double. von Willebrand factor and factor VII increase late in pregnancy, while factor XI usually decreases.[126] In spite of the view that pregnancy is a relatively hypercoagulable state, neither clotting nor bleeding times are outside the normal range. Thus, there is controversy regarding the importance of the known changes in hemostasis. In pregnant women there are a number of conditions that may lead to pathological hypercoagulability and to fetal loss.[127,128] These thrombophilias include antithrombin III deficiencies, protein C and S deficiencies, dysfibrinogenemias, activated protein C resistance, factor V Leiden mutation, and antiphospholipid syndrome.[127]

Cardiovascular Responses to Exercise in Pregnancy

The evolution of advice regarding exercise from physicians to their pregnant patients over the past 50 years has been interesting. Mid-twentieth century advice was based not only on ignorance of the physiology of exercise in pregnancy but also on fear that the cardiovascular fatigue and hyperthermia associated with exercise might harm the fetus. In the 1950s, sports activities were discouraged for pregnant women and only light housework was recommended. But by the 1980s, moderate exercise was recommended by the American College

TABLE 43.3 Coagulation Proteins in Pregnancy[125]

	Increased	Decreased
Procoagulants	I, V, VII, VIII, IX, X	XI
Anticoagulants	Soluble thrombomodulin, TM	Protein S
Adhesive proteins	von Willebrand factor, vwf	
Fibrinolytic proteins	Plasminogen activator inhibitors, pai-1, pai-2	Tissue plasminogen activator, tpa
Placental trophoblast	Tissue factor	Tissue factor pathway inhibitor

Modified from Brenner. Thromb Res *2004;**114**:409.*[125]

of Obstetricians and Gynecologists.[129] While only a half dozen studies have been published, it appears that maternal maximal oxygen consumption ($\dot{V}O_{2max}$) is not different between pregnant and nonpregnant women even with regular weight-bearing exercise. These findings have been summarized.[130] Care providers who might advise against exercise during a normal pregnancy now face an extensive literature on the physiological responses to exercise in pregnancy that show clear benefit. Thus, it is no longer controversial to suggest that moderate, nonexhaustive exercise during pregnancy will bring many health benefits, including less maternal weight gain, reduced risk of preeclampsia, a potentially more efficient placenta (rat), and a heavier baby with a healthy body composition.[130,131]

RESPIRATORY SYSTEM ADAPTATIONS

It is not surprising that the respiratory system must accommodate the energy demands of pregnancy. The single fetus consumes ever increasing amounts of oxygen, and multiple gestations require even more at any corresponding age. Oxygen flow into the maternal blood must then increase not only because of increasing fetal oxygen consumption but also because of increased maternal tissue mass and metabolic demand. Many of the respiratory adaptations have been the subject of published studies and reviews.[132–149] The most profound pregnancy-induced adaptations of the respiratory system arise from two maternal changes:

1. increases in progesterone/estrogen that drive alveolar ventilation during pregnancy;
2. reconfiguration of the chest wall and the elevation of the diaphragm in accommodation of growth of the gravid uterus and mobility of the rib cage.[149]

Hyperventilation

Minute ventilation, $\dot{V}_E$, and alveolar ventilation, $\dot{V}_A$, each increase by about 50% during pregnancy as a result of the central influence of progesterone and perhaps depressed osmolality, augmented Ang II, and AVP.[132,139,141,144,145,147,150] Hyperventilation begins at least by 8 weeks of gestation and depresses alveolar/arterial PCO_2 from its normal resting prepregnant value of ~38 torr (menstrual cycle dependent) to about 30 torr at term; even lower values would not be unusual. The increased alveolar ventilation of pregnancy is maintained mostly by an increase in tidal volume (TV) without much change in respiratory rate. Depressed alveolar carbon dioxide pressure is accompanied by an increase in the partial pressure of oxygen in arterial blood to at least 108 torr early in gestation. Some investigators report a small decrease in PaO_2 to about 104 torr at the very end of term. As the fetus grows and produces more carbon dioxide, the mother must excrete the extra carbon dioxide produced by the fetus in addition to her own. The changes in maternal arterial gas pressures improve the transplacental gradients across the placenta for oxygen, facilitating oxygen flux into the fetal compartment, and for carbon dioxide, facilitating the net carbon dioxide flux from the fetus to the mother. The decreased maternal arterial PCO_2 results in a mild respiratory alkalosis with appropriate renal compensation. Maternal serum pH is maintained at or below 7.45 during pregnancy with a depressed bicarbonate ion level of 18–21 mEq/l.

Lung Volumes and Function

Figures 43.13 and 43.14 show the primary changes in lung volumes and flows that begin after the second half of pregnancy and become pronounced during the third trimester.[132,138,143] These changes include the following:

1. Vital capacity (VC, the amount of gas that can be expelled from the lungs following inspiration to total lung capacity (TLC)) is unchanged.
2. TV (the amount of gas inhaled each breath) is increased by some 40%.
3. Functional residual capacity (FRC, the gas volume remaining in the lungs at rest following exhalation of a tidal breath) is reduced by 20%.

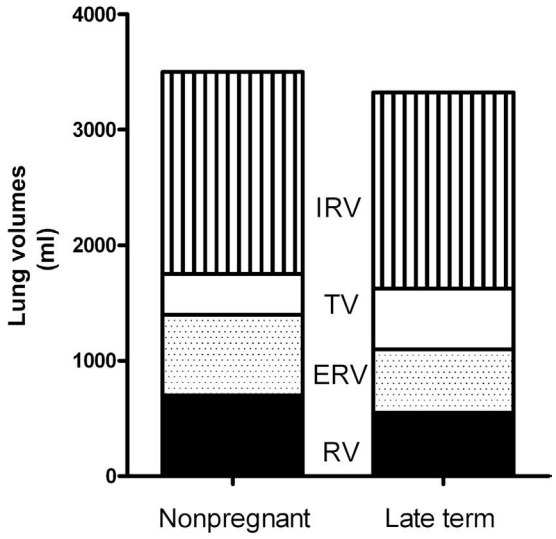

FIGURE 43.13 Lung volumes before and during term pregnancy. IRV = inspiratory reserve volume; TV = tidal volume; ERV = expiratory reserve volume; RV = residual volume. *Source: Data from Ref. 104.*

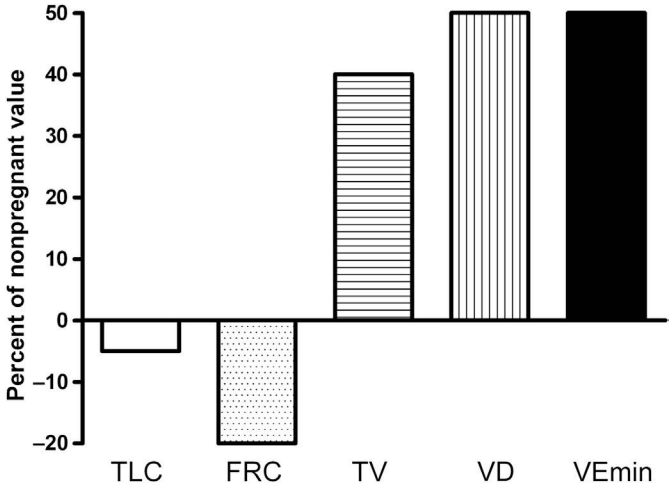

FIGURE 43.14 Maximal percent changes in respiratory parameters during pregnancy compared to the nonpregnant state. TLC, total lung capacity; FRC, functional residual capacity; TV, tidal volume; VD, dead space volume; VEmin, minute volume, rate of expired gas per minute. *Source: Data from Ref. 105.*

The component volumes of the FRC are the expiratory reserve volume (ERV, the volume of gas remaining after a tidal breath that can be exhaled with effort) and the residual volume (RV, the volume remaining in the lung following maximal expiratory effort). Each is reduced approximately equally by some 20%.

4. The sum of all volumes, TLC, is reduced by a modest 5%.
5. Dead space (V_D) increases by about 50%, but the resting dead space to TV ratio (V_D/V_T) does not change because of the increase in TV.

Virtually all respiratory adaptations of pregnancy resolve soon after delivery.[148] Total airway resistance may be reduced during pregnancy, by unknown mechanisms, but overall lung compliance does not change. There are no major changes in standard respiratory functional indices using spirometry-based measurements. Forced expiratory flow in 1 s (FEV_1), mean peak expiratory flow rate (~450 ml/min), and VC are virtually unchanged in U.S. women at sea level.[151] However, ventilatory drive appears to be increased.[140,146]

In 2004 McAuliffe and coworkers reported on respiratory physiological measurements in each trimester of women with singleton pregnancies at sea level (Lima, Peru) and at altitude (Cerro de Pasco/4300 m).[152] They made three interesting findings: (1) nonpregnant and pregnant women alike who lived at altitude had larger lung volumes by about 11 than did sea level women in spite of a 4 cm deficit in height for Cerro de Pasco women, (2) women living at sea level had greater TLC and RV in the third trimester compared to the first trimester, (3) women living at altitude showed a lower FEV_1 in the third trimester compared to the first. These data suggest that pregnancy at altitude demands additional adaptations in respiratory function that are not needed in populations that have lived for generations at sea level.

In normal women, the oxygen cost of breathing is increased in late gestation by some 50%. This is due, in part, to the decreased chest wall compliance that accompanies the reconfiguration of the wall due to the increased intra-abdominal pressure as the growing uterus impinges on abdominal contents and the diaphragm. As pregnancy progresses, the circumference of the abdomen increases, the diaphragm is elevated by some 5 cm, and the chest configuration is altered. One aspect of the reconfiguration is the increase in the average costal angle that accompanies pregnancy earlier than can be explained by increased abdominal pressure with uterine growth. The transverse and anterior–posterior dimensions of the chest increase, which leads to a circumferential increase in the lower thorax of about 5–7 cm.[136,140,146,147]

Diffusing Capacity

The degree to which diffusing capacity of the lung for oxygen changes during pregnancy is controversial. Diffusing capacity for carbon monoxide (DLCO) measurements have been made in women during pregnancy. Because decreasing hemoglobin concentrations in pregnancy affect the measurement of the DLCO, one would expect decreased DLCO values even without any change in the physical attributes of the pulmonary barrier. Milne and coworkers reported an early increase (11 weeks) and a mid-gestation decrease in DLCO.[139] Thus, there may be

changes in barrier function, but further investigation is required to clarify this issue.

RENAL ADAPTATIONS

Overview

Early in pregnancy, well before the human embryo is of significant size, the maternal kidney undergoes dramatic changes in structure, hemodynamic regulation, and tubular handling of solute and water. The primary renal responses to pregnancy have been well documented in previous studies and summarized.[153–158] These well-established changes in the kidney during pregnancy can be briefly summarized as follows:

Glomerular Filtration Rate and Effective Renal Plasma Flow (Figure 43.15)

Glomerular filtration rate (GFR), as measured by creatinine or inulin clearance, increases by approximately 40–65% by the end of the first trimester to a peak of around 180 ml/min[159–162] and is subsequently sustained until term.[161] Effective renal plasma flow (ERPF), as measured by para-aminohippurate (PAH) clearance, also increases by 150–185% above normal at its peak during early pregnancy; it then slowly declines over the second and third trimesters to reach ~10% above the normal nonpregnant level at term.[157] An important caveat applies: a valid comparison of ERPF in normal versus pregnant states via PAH clearance requires that the fractional PAH extraction (EPAH) across the kidney (normally 0.92) remains constant in the pregnant versus nonpregnant kidney.[163] To our knowledge, the renal extraction of PAH has never been measured in human pregnancy but is known to vary up to 20% in various physiological and experimental conditions in other studies.[163]

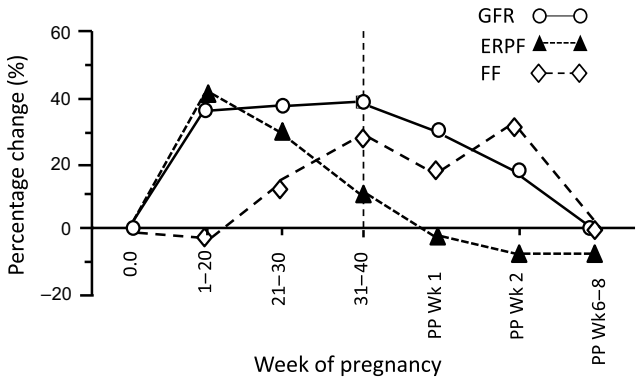

FIGURE 43.15 Changes in renal plasma flow and glomerular filtration rate in normal human pregnancy. Percent changes in glomerular filtration rate (GFR; black line), effective renal plasma flow (ERPF; red line), and calculated filtration fraction (FF; GFR/ERPF) over the course of gestation and early postpartum are shown relative to the nonpregnant state. Data were drawn from published studies based on clearance of inulin or iodothalamate for GFR and on clearance of PAH for ERPF. *Source: From Ref. 157, with permission.*

Blood Urea Nitrogen and Creatinine

Serum urea nitrogen and creatinine levels decrease during pregnancy to values averaging 9.0 and 0.5 mg/dl, respectively, from nonpregnant values of 13.0 and 0.8.[154] A serum creatinine value of greater than 0.8 mg/dl may therefore indicate abnormal renal function in pregnancy.[164]

Protein Excretion

Increases in total urinary non-albumin protein excretion of up to 260 mg/day are normal during pregnancy.[165] Urinary albumin excretion often increases but remains within the normal pregnant range.[157]

Sodium Handling

Renal tubular function is also changed during pregnancy.[155,156,158,166] The filtered load of sodium increases significantly due to the increased GFR. Despite this, a concomitant increase in tubular reabsorption of sodium results in a net retention of up to 1 g of sodium per day.[167,168] Retention of sodium is accompanied by proportional amounts of water, yielding expansion of ECFV up to 1–2 l in the intravascular space plus 4–7 l in the interstitial space.[166]

Renin Release

Renin release and plasma renin activity increase approximately two-fold early in pregnancy, are sustained through the second trimester, and then plateau.[161,169–171] This reflects reduced vascular filling,[172] lower blood pressure, and sympathetic nervous system (SNS) neural activation,[161] as well as hormonal changes.

Water Retention

Pregnancy is also a period of marked water retention.[158,167,168] This reflects sodium-free water, is a consequence of a lowered osmotic threshold for ADH secretion,[173] and yields a mildly lower body-fluid osmolality and serum sodium in normal pregnancy.[173] Intravascular volume expands by around 1–2 l and extravascular volume by approximately 4–7 l.[166]

Glycosuria, Aminoaciduria

Loss of glucose in the urine (glycosuria) is a common finding during normal pregnancy, resulting from increased glomerular filtration and coupled with decreased distal tubular glucose reabsorption.[157,174–178] Similarly, aminoaciduria in normal pregnancy is believed to reflect altered reduced tubular reabsorptive activity.[179]

Structural Changes

Kidneys increase in length and volume during pregnancy, reflecting increased vascular volume and interstitial fluid as well as nephron hypertrophy.[155,158] "Physiologic hydronephrosis" describes the typical pelvic and collecting system dilatation of pregnancy commonly attributed to hormonal influences.[158]

Recent Progress in Understanding Renal Adaptations to Pregnancy

Recent investigations now address the cellular and molecular mechanisms that underlie renal modifications associated with pregnancy.[154,180]

Renal Hemodynamic Changes in Pregnancy

A number of studies in normal rat,[153,181,182] rabbit,[183,184] sheep,[185] and human[164,186–188] pregnancies have established that ERPF and GFR progressively increase over the course of gestation as renal vascular resistance (RVR) declines. In women, GFR peaks in the first trimester and maintains elevated levels of 40–60% above nonpregnant levels until delivery.[186,189] ERPF also increases early but plateaus after 8–12 weeks of gestation. In the rat, both ERPF and GFR peak in midgestation at ~30% above virgin levels.[159] These hemodynamic changes are induced by maternal factors, as they do not require the presence of the feto-placental unit. Similar hemodynamic responses are observed in pseudopregnancy.[190,191]

Chapman and colleagues recently reexamined the time course of renal hemodynamic changes in normal human pregnancy ($n = 13$), using serial measurements of women in the left lateral decubitus position, employing gold standard classic methods (clearances of inulin and PAH), and including a preconception baseline and postconception measurements spanning 6–36 weeks gestation.[161] In agreement with prior work, ERPF was increased by six weeks, reached a maximum at eight weeks, and declined slightly by 36 weeks. RVR decreased in a mirror-image pattern as expected.[161] In contrast, GFR rose in parallel to renal plasma flow over about the first 6 weeks and then continued to increase until 12–24 weeks. Peak renal plasma flow increased to ~50% above nonpregnant levels at 8 weeks; the GFR increased by an average of ~27% by 12 weeks and ~55% by 36 weeks. Filtration fraction (fraction of RPF filtered at the glomerulus) remained below prepregnancy values throughout gestation. In a separate study, Roberts and coworkers estimated 72% and 45% increases in RPF at 16 and 36 weeks gestation, respectively.[192] They also mostly generally agreed with the Chapman study with a ~55% increase in GFR at both time points in normal pregnancy ($n = 11$). Based on a review and resynthesis of data from multiple studies, Odutayo and Hladunewich reached similar conclusions (Figure 43.15).[157]

Mechanisms of Increased GFR: Does Hyperfiltration in Pregnancy Convey Risk of Renal Injury?

Understanding the increased GFR in pregnancy is important in considering whether pregnancy can induce injury to the normal kidney.[159] GFR is determined by the average net filtration pressure within the glomerular capillary (Figure 43.16). An increase in glomerular capillary pressure (Pgc) is the typical mechanism underlying glomerular hyperfiltration in pathological states of nephron loss when sustained chronically.

The net filtration pressure at any point along the glomerular capillary is determined by the algebraic sum of hydrostatic and osmotic pressures across the filtration barrier. The net hydrostatic pressure is the pressure difference (ΔP) between the capillary pressure (Pgc) and the tubular hydrostatic pressure (PT); the oncotic pressure component is determined by plasma oncotic pressure (πgc) alone. The net hydrostatic pressure across the capillary wall favors fluid flow out of the capillary and into the renal tubule; at any point along the capillary, ΔP is offset by the oncotic pressure that opposes water flow from the capillary. The formula for glomerular filtration rate can be expressed as:

$$\mathrm{GFR} = (\Delta P - \pi\mathrm{gc})\,\mathrm{Kf}$$

where Kf is a filtration constant that incorporates the filtration surface area and intrinsic hydraulic conductivity of the glomerular capillary wall. The average ultrafiltration pressure is the summation of pressures along the capillary length, represented in Figure 43.16 as the shaded area. An additional factor influencing GFR is the renal plasma flow, which determines the rate of rise of πgc along the capillary length (Figure 43.16). Thus, an increase in renal plasma flow, when changed in isolation, slows the rate of rise of πgc, enhancing the net filtration pressure. When comparing across species, the degree to which changes in renal plasma flow affect GFR is believed to differ substantially in rats versus humans. Under basal conditions, the rat kidney is in "filtration pressure equilibrium," that is, the rise in oncotic pressure leads to cessation of filtration at a point prior to the end of the capillary length (Figure 43.16; solid line for πgc). Plasma flow has a large influence on GFR in the rat because flow increases (e.g., dotted line in Figure 43.16) allow filtration to occur along a greater fraction of the capillary length, thereby substantially increasing filtration surface area. In the human kidney, where "filtration pressure disequilibrium" pertains (Figure 43.16), filtration occurs along the entire capillary length under

basal conditions; thus, increases in renal plasma flow alone cannot recruit additional filtration surface area in the normal nonpregnant human kidney and thus have a more modest effect on GFR.

Glomerular Hemodynamics in Rat Pregnancy

Micropuncture studies in the rat, where GFR is substantially dependent on renal plasma flow, have shown that the pregnancy-associated increase in single-nephron GFR is accounted for by the increase in renal plasma flow.[193,194] Directly measured pressure in the glomerular capillary, Pgc, was unchanged by pregnancy in rats, indicating that: (1) hyperfiltration in rat pregnancy does not require glomerular capillary hypertension, and (2) renal vasodilation involves proportional reductions in resistances in the afferent (RA) and efferent (RE) arterioles.[194] Because a value for the filtration coefficient, Kf, could not be determined, a change in Kf could not be excluded in the normal pregnant rat.

To directly address the potential for renal injury with repetitive pregnancies, Bayliss and Rennke tested the long-term effect of five consecutive closely spaced pregnancies in normal Munich-Wistar rats.[195] No increase in Pgc was apparent as compared to age-matched virgins, and no adverse effects of repeated pregnancies on kidney function or structure were observed.[195] Although Reckelhoff found a greater tendency for renal functional decline in Sprague Dawley rats after eight successive pregnancies, no proteinuria or renal structural pathology was noted.[196] Studies in the rat indicate that the renal adaptation to pregnancy does not involve intracapillary hypertension, that pregnancy-induced changes fully resolve during the postpartum period, and that renal hyperfiltration during repetitive pregnancies does not appear to impose a risk of renal injury.[153]

Intraglomerular Hemodynamics in Human Pregnancy

Renal micropuncture is not possible in humans. Furthermore, since rat intraglomerular hemodynamics differ significantly from the human (see previous discussion), interspecies inferences are potentially unreliable. Therefore, recent investigations have attempted to estimate determinants of GFR in human pregnancy using computerized mathematical modeling approaches. ERPF, GFR, πgc, and clearances of neutral dextrans across a range of sizes were measured and combined with estimates for unmeasurable parameters such as mean pressure of the glomerular capillary and Kf.[189,192,197,198] Roberts and coworkers suggested that the mid-gestation increase in GFR of 55% could be fully explained by increased renal plasma flow; whereas the late GFR increases are likely due to additional factors, including a reduction from peak renal plasma flow as well as a fall in πgc of 5.9% in early gestation and 19.7% in late gestation.[192] Results did not support glomerular capillary hypertension as the primary driver for increased GFR across a range of feasible Kf and Δ*P* values. In response to technical criticisms, this group repeated their studies with improved protocols using two different mathematical models.[197] They again concluded that the 38% increase in GFR observed in late pregnancy reflected increased renal plasma flow, reduced πgc, and increased Kf with no increase in the net driving pressure (Δ*P*) across the capillary wall (Figure 43.16).

Postpartum Glomerular Hemodynamics in Human Pregnancy

Postpartum observations in women add perspective to our understanding of the mechanisms that lead to the resolution of renal adaptive responses during pregnancy and to the timing of these mechanisms. Lafayette and coworkers studied women immediately after Cesarean delivery, using computer models of glomerular

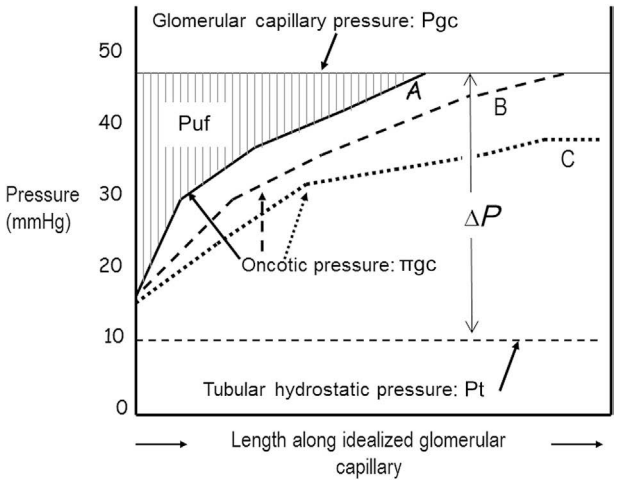

FIGURE 43.16 Regulation of glomerular ultrafiltration. At any point along the length of a glomerular capillary, the force driving filtration is a function of net hydrostatic pressure favoring filtration (Δ*P* = glomerular capillary pressure Pgc minus the opposing intratubular pressure Pt) less the net colloid osmotic pressure (πgc) which also opposes filtration. Increased GFR can be induced by increase in plasma flow, by decrease in oncotic pressure, or by increase in glomerular capillary pressure. GFR is proportional to net ultrafiltration pressure (Puf), defined as the area dilineated by the, Pgc and πgc profiles (e.g., shaded area for πgc profile A). "Filtration equilibrium" refers to an oncotic pressure profile (e.g., solid line A or dashed line B) where filtration ceases prior to the end of the capillary. The rat operates in filtration equilibrium under basal conditions: With increased plasma flow rate, the πgc profile can shift to e.g., line B or line C; The parallel increase in Puf area reflects the increase in GFR due to elevation of plasma flow. In "filtration pressure disequilibrium" (πgc profile C), πgc rises sufficiently slowly that it never reaches Δ*P*; thus filtration continues to occur along the entire capillary length. Humans, in contrast to rat, are believed to operate basally in filtration disequilibrium. An increase in RPF thus cannot increase filtration surface area and thus has much more limited impact on GFR. *Source: Adapted from Ref. 158.*

parameters.[199] They reported a persistent glomerular hyperfiltration without hyperperfusion; the former was attributed entirely to the persistent decrease in plasma oncotic pressure after delivery. The same group recently reported similar studies at 1 day and at 2 weeks postpartum in 22 uncomplicated pregnancies.[189] On the first day after delivery, the model again supported reduced capillary oncotic pressure as the predominant factor in maintaining a 41% increase in GFR. By 2 weeks postpartum, GFR was still increased by 20% even though renal plasma flow and oncotic pressure had returned to normal nonpregnant levels; thus, only unmeasurable parameters could be postulated to maintain GFR, such as an increase in ΔP, Kf, or both. Milne and coworkers reported that GFR and renal plasma flow had returned to prepregnancy values by 4 months after delivery.[197]

Despite these careful efforts to enhance our knowledge of renal hemodynamic changes in human pregnancy, caution is in order. The unverified assumption of constant EPAH underlying estimation of ERPF changes by PAH clearance has already been noted; it remains feasible that the apparent ERPF decline following the midpregnancy peak reflects progressively reduced tubular extraction of PAH with consequent underestimation of ERPF. Inferences about ΔP necessarily involve modeling based on rat data and require assumptions that cannot be validated at the present time. Moreover, these theoretical considerations do not address the possibility that the glomerular hypertrophy of pregnancy, via increased glomerular capillary length, might create glomerular filtration pressure equilibrium in the human kidney as in the rat. This would enhance the flow dependence of GFR. Glomerular hypertrophy might also increase filtration surface area. Either the pregnancy-induced increase in capillary length or the increased infiltration area could obviate the need to postulate for either increased PGC or increased permeability (Kf). It should also be emphasized that while renal adaptations in pregnancy are normally safe, even modest impairment of renal function in human pregnancy (GFR < 50 cc/min) is known to impose a substantial risk of further renal functional loss.[200–202] Finally, it is not yet known in humans whether successive pregnancies increase risk of renal injury to the human kidney.

Glomerular Capillary Permselectivity to Neutral Macromolecules in Human Pregnancy

An estimate of glomerular capillary wall porosity to macromolecules in human pregnancy has been made, utilizing fractional clearance of neutral dextrans spanning a wide size range. This measurement is independent of hydraulic permeability. Neutral dextrans are not restricted in their passage across the glomerular filter by charge or configurational factors, nor are they absorbed or secreted by the renal tubule. Thus they are classically used to assess the glomerular size-selective barrier function. It was a surprise when Lindheimer's group found reduced fractional clearance of dextrans at the lower size ranges during late gestation.[192,197] This finding points to an altered structure of the glomerular capillary membrane. Their findings could be explained by (1) an increase in molecular movement through a nondiscriminatory "shunt" pathway or (2) an increased range of pore sizes in the late-gestation glomerular capillary wall.[197]

Urinary Protein and Albumin Excretion in Pregnancy

Protein that is present in normal urine derives from filtered protein that escapes proximal tubular reabsorption and/or from secreted protein, primarily Tamm–Horsfal protein formed by the distal nephron segments. Urinary albumin excretion rate can increase due to a decrease in the discriminatory capacity (permselectivity) ion of the glomerular capillary wall or to a reduced tubular reabsorption of normally filtered proteins. The latter leads to an increased excretion of non-albumin low molecular weight (MW) proteins—below MW 10,000 Da—together with small increases in albumin. Several studies report that total urinary protein (but not albumin) increases by about two-fold by late gestation.[197,203–205] Higby and coworkers reported an average total protein excretion of 117 mg/day with an upper 95% confidence limit of 260 mg/day in 270 healthy pregnant subjects.[165] In normal pregnancy, albumin excretion increased minimally to an average of 12 mg/day with an upper limit of 29 mg/day, thus remaining within the normal nonpregnant range. Collectively, this suggests that proximal tubular reabsorption of normally filterable low MW proteins (but not albumin) may be selectively reduced. Alternatively, increases in plasma levels of filterable smaller-molecular-weight proteins or increased tubular synthesis/secretion of Tamm–Horsfall protein could account for the increase.

Changes in albumin excretion in human pregnancy remain controversial. Misiani and coworkers measured albumin by radioimmunoassay in healthy pregnant women and found a reduction in urinary albumin excretion to below nonpregnant levels throughout gestation.[204] This was in distinct contrast to cases with pregnancy-induced hypertension, where albumin excretion began increasing by 28 weeks gestation.[204] Milne and coworkers found that urinary albumin was higher than nonpregnant levels in 11 healthy women in late gestation and persisted at 4 months postpartum.[197] But, once again, these changes were within the normal nonpregnant range. Furthermore, whereas the increase in glomerular permselectivity to small neutral dextrans fully normalizes by 12 weeks postpartum, urinary albumin remains at late-pregnant levels.[192,197]

These findings suggest that additional factors favoring increased albumin excretion may be operative. Of note, increased urinary amino acid excretion during pregnancy has been documented and could indicate a more generalized impairment of proximal tubular reabsorptive capacities.[179]

Structural Changes of Kidney and Genitourinary Tract

During the course of normal pregnancy, the kidneys enlarge by up to 30%. In one large autopsy series, kidney weights during or shortly after delivery (ranging 300–310 g) exceeded the nonpregnant norms; glomerular size, but not cell number, was increased.[206] X-ray[155,158] and ultrasound[207] studies have shown that kidneys increase in length by approximately 1 cm during human gestation. In rats, kidney dry weight during pregnancy has been found to increase along with increases in proximal tubular length, changes resembling those in compensatory renal hypertrophy.[208,209] In other studies, increased renal weight was attributed to renal water content.[210] These changes, seen in >90% of gravidas, are associated with dilation of the renal calyces, pelvices, and ureters,[155,211–213] the latter often more marked on the right due to dextrorotation of the uterus.[214] Dilation of the renal calyces is accompanied by ureteral smooth muscle hypertrophy and connective tissue hyperplasia. The mechanisms that lead to these remarkable changes are unknown. Of note, renal structural changes may persist up to 4 months postpartum.[212] It is unknown whether increased compliance of these structures in pregnancy reflects the actions of relaxin on smooth muscle, as described for the vasculature and uterus in the rat,[104,215] the actions of relaxin to alter connective tissue structure of the rodent cervix,[216] or other hormone-related mechanisms.

The Kidney and ECFV and Blood Pressure Regulation in Pregnancy: The Relaxin Paradigm in the Pregnant Rat

Investigators have long struggled to understand how the regulatory system for renal sodium reabsorption and ECFV is co-opted to retain sodium and produce a positive sodium balance, while avoiding the expected feedback suppression of sodium retention. It is not clear, for example, whether observed renal changes are secondary to systemic signals, or whether they are initiators of systemic responses. One view holds that primary systemic vasodilatation induces secondary renal sodium and water retention, driving the expansion of ECFV and blood volume that is characteristic of pregnancy from its early stages.[48,154,217] An alternative view is that the kidney vasodilates early in pregnancy and acts as an arteriovenous shunt, which secondarily activates systemic sodium/water retaining pathways.[154] Neither view alone fully reconciles renal responses in pregnancy with known principles of hemodynamic regulation. In the nonpregnant state, the introduction of extrarenal vasodilation leads to vasoconstriction of renal circulation as a key feature of its response to circulatory underfilling. In contrast, the renal circulation undergoes a remarkable vasodilation from early in pregnancy, concomitantly with systemic vasodilation.[48] We propose another option: that systemic and renal resistances are reduced proportionally by a common stimulus.

Candidate Vasoactive Mediators in Rat Pregnancy

Attempts to identify specific systemic or locally active vasodilators that account for all the hemodynamic changes in pregnancy have failed. Factors such as estrogen, progesterone, prolactin, and vasodilatory prostaglandin species may be important but do not explain all of the vasodilator actions needed for adaptation to pregnancy.[154,155] However, in elegant studies in the pregnant rat, Conrad and colleagues have documented a major role for relaxin, a TGF-beta family member with systemic vasodilating capacity.[154,218] Relaxin is produced by the corpus luteum, and levels rise in temporal association with the early progressive fall in systemic vascular resistance as pregnancy progresses.[219] In the nonpregnant rat, Conrad and coworkers found that relaxin reproduces many of the characteristics of the pregnant vasculature: reduced vasomotor tone, increased global arterial compliance, modification of vascular extracellular matrix structure, and reduced vascular stiffness (measured independently of tone).[117,175,219,220] These effects of relaxin were found in small renal arteries[117] and the uterine artery of the rat,[157,220,221] as well as in numerous arteriolar, capillary, and postcapillary venular sites throughout the body.[215] In the pregnant rat, relaxin was shown to mediate maternal renal vasodilation and hyperfiltration,[218] to reduce the myogenic activity of small renal arteries, and to reduce their reactivity to Ang II.[222]

Subsequent work by this group elucidated downstream roles of endothelin[223] and NO[224,225] in mediating the renal vasodilation and hyperfiltration effects of relaxin in rat pregnancy.[154] Specifically, the vascular effects of relaxin, normally acting via its RXFP1 receptor,[157,226] were mediated by endothelin 1 (ET1), through its endothelin B (ET_B) receptor to stimulate NO production.[117,223,227] Moreover, relaxin was found to increase the ET_B:ET_A receptor ratio. In recent work, relaxin was additionally shown to upregulate[157,228] and activate vascular gelatinases,[219] which likely convert Big ET1 to a smaller version, ET1-32.[157,229,230] Relaxin was also found to require both vascular endothelial growth factor and placental growth factor in order to attenuate myogenic constriction in rodent renal arteries and human

subcutaneous arteries.[157,231] Jeyabalan and coworkers showed that increases in the vascular gelatinase MMP2 are essential for relaxin-induced renal vascular effects, possibly via conversion of Big ET1 to ET1-32.[230] This work establishes a novel mechanism underlying classic pregnancy-induced renal hemodynamic changes in the rat, and concomitantly unveils a unique MMP-2 gelatinase-linked vasodilatory pathway with potentially broad relevance for renal vascular regulation.

Renal Tubular Changes during Pregnancy

Before considering the implications of this novel vasodilatory mechanism for pregnancy-associated hemodynamic adaptations, it is important to first revisit recently recognized factors acting on tubular sodium handling during pregnancy. There are both antinatriuretic (sodium ion retentive) and natriuretic (promoting sodium loss in urine) forces that are present during pregnancy. What allows the former to predominate is not clear, but pregnancy is unequivocally associated with net renal sodium retention.[155,158] The positive balance in women over the course of gestation averages 2–6 mEq of sodium daily with cumulative gains of 500–950 mEq.[155,158] Studies in humans using lithium clearance as a sodium marker document enhanced proximal tubular sodium reabsorption in pregnancy.[232] Micropuncture studies in the rat have also shown that sodium delivery to the end of the accessible proximal tubule is normal in pregnancy, indicating that the excess glomerular filtrate is fully reabsorbed proximally.[233] Paradoxically, this increased reabsorptive work in the proximal tubule is not accompanied by increases in renal microsomal sodium-potassium-adenosine-triphosphatase (Na-K-ATPase) activity, the primary energy source for tubular reabsorption throughout the nephron.[234]

Recently, a mechanical factor has been shown to influence proximal tubular sodium retention in pregnancy. Khraibi and coworkers documented a reduction in intrarenal interstitial hydrostatic pressure (RIHP) in the pregnant rat,[235–237] a feature that should enhance sodium reabsorptive capacity.[238] In the nonpregnant rat, increased renal perfusion pressure or volume expansion is associated with an increase in RIHP and subsequently natriuresis[238]; if the increase in RIHP is prevented by stripping the renal capsule, no natriuresis ensues. In pregnant rats, basal RIHP was found to be low and failed to increase normally following raised renal perfusion pressure or saline expansion[236,237]; however, an expansion of the ECF volume with saline did induce a normal natriuretic response, while an increase in perfusion pressure did not. It is useful to consider how reduced basal RIHP might influence sodium-retaining forces (elevated Ang II, norepinephrine) versus the natriuretic forces (variably increased ANP[161] and reduced renal cortical Na-K-ATPase activity and abundance[234]). Low RIHP would be expected to reduce the passive intercellular back leak of sodium (with Cl^- and H_2O) in the normally leaky proximal tubule, causing more reabsorbate to be retained and thereby passively enhancing net reabsorptive flux. Sodium reabsorptive activity of Ang II and SNS traffic in the proximal tubule could thereby be substantially amplified. In contrast, the natriuretic response to ANP was blunted in pregnant rats.[239] RIHP would seem less likely to influence more distal tubular segments due to their greater water impermeability.

In the distal tubule, additional factors are believed to modulate sodium/H_2O retention during pregnancy. The mineralocorticoid hormone, aldosterone, which is secreted from the adrenal cortex, is essential for normal renal sodium and potassium balance, and thus indirectly regulates blood volume. The rate of aldosterone secretion and its consequent plasma levels are increased in normal gestation, frequently approaching levels associated with primary hyperaldosteronism.[155,161] In addition, it is known that a specific mutation in the mineralocorticoid receptor leads to enhanced hypertension during pregnancy.[240] Experiments in sheep suggest that loss of aldosterone or cortisol lead to a decrease in blood volume during pregnancy.[240] The sex steroid progesterone has both agonistic and antagonistic activity at the mineralocorticoid receptor. However, in vivo, efficient renal enzymes metabolize progesterone, effectively protecting the mineralocorticoid receptor.[241,242] However, pregnancy also increases intrarenal enzymes that metabolize progesterone.[243,244] The net effect of hormone action is not yet clear. Nonetheless, the kidney effectively retains 2–6 mEq sodium daily during gestation, establishing ECF volume increases of 5–7 l. The increased levels of aldosterone appear necessary to achieve this critically important adaptation.

The relaxin story offers a potential solution to one major pregnancy conundrum: how to entice the kidney to generate and maintain an expanded ECFV in anticipation of future feto-placental needs. The key elements of the solution in the rat are now discernible.[154] First, a single systemic hormone (relaxin) simultaneously mediates vasorelaxation at multiple systemic sites and in the kidney. Second, the downstream vasodilatory effect of relaxin is ensured by upregulation of a classic vasoconstrictor system (ET1) while concurrently enhancing its vasodilatory activity via an increase in ET_B receptor relative to ET_A receptor. Third, the final common pathway of this hormonal system is NO, a potent vasodilator that also has known effects on capillary permeability and structure. Finally, relaxin has been shown to have widespread effects on tissue

structure to increase compliance, a recurring theme in tissue responses to pregnancy.[154,215,245]

Proposing an Integrated View of the Renal Response to Pregnancy

The actions of relaxin in the pregnant rat create a physiologically unique set of intrarenal conditions. We propose the following canonical model to explain many of the features of mammalian pregnancy and associated changes in the kidney. First, primary vasodilation of both systemic and renal vasculatures generates systemic vascular underfilling via increased vascular capacity, a change normally expected to activate central venous stretch receptors. The increased SNS traffic, together with its subsequent stimulation of renal renin release, initiates renal salt and water retention; if these stimuli were unopposed, they would simultaneously cause both pre and postglomerular renal vasoconstriction and renal tubular sodium/H_2O reabsorptive capacity. However, Relaxin-ET_B receptor-induced NO concomitantly mediates resistance to the pressor effects of Ang II and norepinephrine. This ensures that both renal preglomerular and postglomerular vasodilation are sustained and proportional, maintaining normal glomerular capillary pressure. In the systemic circulation, resistance to pressors ensures that the reduced stretch at central venous receptors cannot be relieved via the usual compensatory pressor actions of Ang II and SNS to reduce the volume capacity of the vascular tree. Thus, the vascular underfilling signal is perpetuated even as ECFV increases. In the kidney, this persistent relaxin-mediated preglomerular vasodilation leads to greater transmission of aortic mean arteriolar pressure (MAP) to the afferent arteriole. Thus, the lower systemic blood pressure can now sustain a normal afferent arteriolar pressure, thereby shifting the renal set point downward to accept and sustain a lower MAP. In effect, despite systemic vascular underfilling, preglomerular vasodilation that is resistant to Ang II and norepinephrine permits the kidney to accept the lower MAP of early pregnancy as "normal". The concomitant resistance to ANP (see previous discussion) suggests that its potential to counteract these forces is effectively neutralized.

As outlined earlier, the renal tubular sodium retention limb of the feedback loop remains intact. This may have important effects on the behavior of the macula densa to regulate GFR in pregnancy. Normally, elevated GFR, despite an increased absolute proximal reabsorption (i.e., glomerulo-tubular balance), will also deliver excess sodium/H_2O to the thick ascending limb. The higher thick ascending limb flow rate would limit NaCl capture and create a higher concentration of sodium chloride sensed by the specialized epithelial cells of the macula densa. This stimulus would normally activate tubular glomerular feedback, causing macula densa cells to release basolateral ATP and activate afferent arteriolar constriction at a discrete sphincter-like site located just within the glomerulus. The outcome would be to reduce Pgc and GFR.[246] In contrast, the pregnant state is accompanied by normal fluid/sodium delivery measured in the late proximal tubule and is compatible with the view that, despite high GFR, stimulated sodium reabsorption proximally—together with low RIHP/reduced back leak—prevents any increase in [NaCl] at the macula densa.[233] The latter is thus rendered blind to the high GFR of pregnancy. This is precisely compatible with the observations of Baylis and Blantz that macula densa function in pregnancy is reset to a higher level of GFR but operates normally around that set point.[233]

Another factor of potential importance in promotion of proximal tubular reabsorptive capacity and modifying macula densa activity during pregnancy is proximal tubular hypertrophy. In early chemically induced diabetes in rats, Thompson and coworkers have provided compelling data to support the idea that hyperglycemia-mediated proximal tubular hypertrophic growth directly enhances sodium reabsorption, reduces [NaCl] at the macula densa, induces afferent-sphincter dilation, and causes a compensatory increase in GFR.[247,248] According to this view, tubular hypertrophy is a primary event that directly leads to enhanced proximal reabsorption and glomerular hyperfiltration by activating (rather than silencing) the macula densa. Evidence in the pregnant rat that proximal tubule length is increased lends support to the potential for proximal-tubular-growth-dependent initiation and perpetuation of glomerular hyperfiltration in pregnancy.[208,209] Of note, hyperfiltration due to proximal hypertrophy/hyperabsorption in diabetes is typically dependent on increased glomerular capillary pressure. In pregnancy, the Ang II pressor resistance of the postglomerular capillary would prevent increased Pgc and require additional intraglomerular changes to achieve GFR elevation.

Mediators of Renal Hemodynamic Adaptations in Human Pregnancy

Available early data based on maternal relaxin levels in human pregnancy suggested that relaxin, at least in circulating form, may mediate the systemic and renal vasodilation.[154] However, the similarities in hemodynamic responses of rat and human suggest that there will be homologous systemically active vasodilatory molecules and common downstream pathways in human pregnancy. In support of that, there is limited evidence that NO may be increased in pregnant women.[161,249] Goodrum and coworkers found higher arginine and NO production in mid-gestation.[249] On the other hand, von Mandach and coworkers found NO increased predominantly in the fetal compartment.[250] Similarly, Schiessl and coworkers detected no changes in either NO metabolites or cGMP levels in plasma or urine over the course of gestation in 49 healthy pregnancies.[251] In the first

study to carefully control for dietary nitrate and nitrite, Conrad and coworkers found no increase in whole body NO production but marked increases in cGMP production during pregnancy, especially during the period of rapid vasodilatation in the first trimester.[252] Recent work has shown that human subcutaneous arteries respond to relaxin via molecular pathways comparable to those of rat small renal arteries.[231] More compellingly, in pregnant women who were recipients of donated eggs and who therefore lacked a corpus luteum (the dominant source of relaxin in human and rat pregnancy[224]) and measurable circulating relaxin, glomerular hyperfiltration of early pregnancy was attenuated.[226,253,254] As these intriguing results do not exclude participation of other corpus luteum factors, the question of whether and how relaxin contributes to the human renal and hemodynamic adaptations to pregnancy remains unanswered.

Role of Angiotensin 1–7 and/or Angiotensin AT2 Receptors

If relaxin is not a mediator of pregnancy-associated hemodynamic change in human pregnancy,[158] we wonder whether there is a human molecule that is equivalent in action to relaxin. (One would expect multiple layers of regulation of blood volume and blood pressure during pregnancy.) Brosnihan and coworkers have recently reported increased levels of the vasodilator Ang 1–7 in plasma and urine of normal pregnant women.[255–257] This vasodilatory metabolite is formed via conversion of Ang II by angiotensin converting enzyme 2 (ACE 2) or by a two-step reaction converting Ang I to Ang 1–9 by ACE2, then to Ang 1–7 by ACE (Figure 43.17). Also, in pregnant rats, increased renal and urinary levels of Ang 1–7 were observed, together with enhanced ACE 2 expression in kidney.[256] These observations suggest a role for vasodilatory components of the renin-Ang II system in human and rodent pregnancy. It is interesting to speculate that, whereas relaxin co-opts the endothelin pressor system and converts it to vasodilatory action in the pregnant rat, the Ang II system might be co-opted to a vasodilatory system in human pregnancy, potentially via increased Ang 1–7, via shift to AT_2 Receptor > AT_1 Receptor ratio, and/or via enhanced Ang II-induced endothelial vasodilatory factors. Evidence already available indicates functional resistance to AT_1R in pregnancy. Like the upregulated endothelin ETB receptor in rat pregnancy, AT_2 Receptor-mediated vasodilation is linked to bradykinin and NO,[258] and thus its upregulation in pregnancy could potentially mimic the relaxin role in the rat. The relaxin paradigm in the rat may provide a valuable template for reassessing the human response to pregnancy.

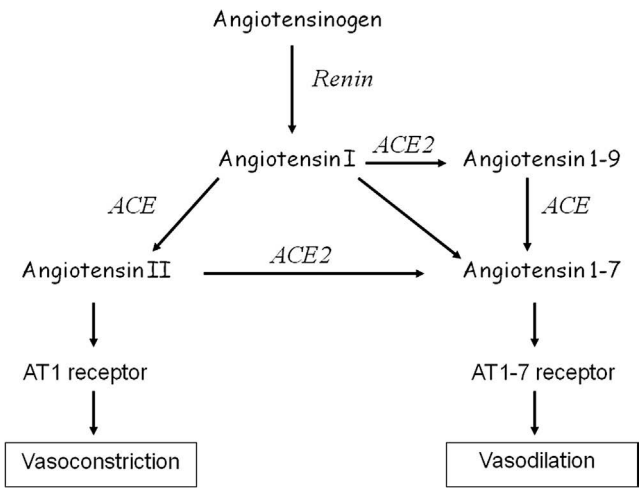

FIGURE 43.17 Enzymatic pathways for formation of the vasodilator angiotensin 1–7. Ang 1–7 can be formed from either Ang II by the action of angiotensin converting enzyme ACE2 or from angiotensin I via a two-step reaction requiring ACE 2 followed by ACE. Ang 1–7 has been reported to increase in the 3rd trimester of normal pregnancy, whereas levels in preeclamptic pregnancies were significantly reduced as compared to normal pregnancy. *Source: From Ref. 256.*

PHARMACOKINETICS OF PREGNANCY

Because of ethical concerns associated with the experimental administration of drugs during pregnancy, less is known about drug pharmacokinetics for pregnant women than for nonpregnant women. However, the application of standard pharmacokinetic principles has provided insight as to how drug handling and metabolism are different in women who are pregnant.

The absorption of a medication from the gastrointestinal tract may be altered during pregnancy. In general, gastric emptying and small intestine motility are reduced in pregnancy, in part due to effects of progesterone.[259–270] In addition, intravascular fluid volume and total body water increase during pregnancy, augmenting the volume of distribution—the body fluid volume within which a substance distributes—for any dissolved drug.

Plasma protein concentrations, including albumin, decrease in pregnancy. As a result, there is less protein available for binding drugs, and the plasma concentrations of albumin-bound drugs decrease.[271,272] However, because less drug can be bound by albumin during pregnancy as its concentrations decrease, an increased concentration of unbound "free" drug becomes available to produce an effect. Thus, while a drug may have a lower concentration in the blood because of low protein binding, more of it may become available to cells.

Several steroid hormones, including estrogens and progesterone, increase during pregnancy. The increasing concentrations of steroid hormone levels can displace drugs from their bound state on albumin resulting in increased concentration of free drug while decreasing the drug's biological half-life.[272] Thus, the drug may

have a greater effect because of its higher concentration at the cell but for a shorter duration. This is because an increased concentration of circulating free drug facilitates its metabolism and excretion. The overall magnitude of these effects is not known for most drugs.

Along with increases in total body water, total protein and fat mass generally increase during pregnancy.[273] The increase in total fat mass provides a larger volume of distribution for lipophilic drugs, those that dissolve easily in lipids. However, the increase in the volume of distribution for lipophilic drugs does not appear to be of great clinical significance for most drugs.

Most medications are metabolized in the liver to forms that are then excreted by the kidneys. Several enzymes of the hepatic cytochrome P-450 system are induced by estrogen and progesterone, resulting in a higher rate of metabolism for some medications, while other enzymes are competitively inhibited by estrogen and progesterone, leading to decreased metabolism and impaired elimination.[274–276] Maternal cardiac output, renal blood flow, and GFR all increase substantially during pregnancy.[164,188,277] These increases may lead to an enhanced elimination of drugs that are normally excreted unchanged. An example of enhanced elimination during pregnancy is penicillin.

Drugs move from the maternal circulation to the fetal circulation mainly by diffusion across the placenta.[278–280] Their rate of transfer depends on their lipid solubility and size. Polar hydrophilic drugs cross the placenta slowly. Highly protein bound drugs, highly charged drugs, and large MW drugs also cross the placenta slowly. On the other hand, lipophilic medications move across the placental barrier to the fetus rapidly because they have the entire trophoblastic surface area available for diffusion. Their drug transfer is influenced by maternal and fetal placental blood flows. Ethanol is an example of a drug in this category. Protein binding is an important consideration for the transplacental transport of lipophilic drugs too because it influences the amount of free drug available for diffusion.

Even though the fetal liver is metabolically active,[281] the metabolic enzyme activity of the immature fetal liver metabolic is low. In addition, some 50% of the fetal blood flow from the placenta bypasses the fetal liver via the ductus venosus and travels directly to cardiac and cerebral circulations. As a result the fetus metabolizes drugs less rapidly than the mother. The fetal liver can metabolize drugs by sulfate conjugation and glucuronidation; such drugs then undergo renal excretion into the amniotic fluid. Drugs in the amniotic can be reabsorbed into the fetal circulation. This process can contribute to drug accumulation in the fetus. Drug metabolites are often ionized, impairing diffusion. As a result, they can accumulate within the fetus as a result of "ion trapping". Fetal blood is slightly more acid than maternal blood. Lipophilic, nonionized drugs that are weakly basic, move by diffusion back and forth across the placenta in a balanced fashion, driven by their concentration gradients. On the other hand, acidic drugs that are ionized in the more acidic fetal plasma favor movement from the mother to the fetus.[282] The placenta itself contains many metabolizing enzymes.[282,283] Some drugs are metabolized by the placenta and are converted into either active or inactive metabolites, depending on the drug in question. In summary, the adaptations to pregnancy made in a woman's body, combined with the biological contributions of the placenta and fetus, alter the distribution and metabolism of drugs compared to the nonpregnant state.

CONCLUSION

Looking backward in time is valuable because it generates an appreciation for the progress that has been made in understanding the physiology of pregnancy. It is clear that the concomitant development of precise physiological measurements of the actions of the cardiovascular, renal, pulmonary, and endocrine systems allowed the field to blossom over the past four decades. Nevertheless, one cannot assess the gains made in this field without a sense of frustration for its slow progression compared to other fields of human biology and medicine. Studies on pregnancy have been relatively few. There is a driving reason for that to change in the near future. It has recently become clear that adaptations to pregnancy are key to determining the developmental integrity of the growing embryo and fetus. Maternofetal nutrient flow plays a major role in determining the risk for chronic disease in a woman's offspring and thus the health of each and every member of the population. Thus, maternal health and the appropriate adaptation to pregnancy will determine the health of the next generation.

In addition to the numerous loose ends that need to be solved in every area of physiology as noted in each section of the chapter, intense research is needed in three major additional areas: (1) the role of maternal body composition and metabolism in regulating adaptations to pregnancy and providing nutrients for offspring; (2) the mechanisms by which the maternal milieu signals and regulates placental growth and function; and (3) the molecular mechanisms by which steroid and protein hormones modify maternal organ structures and their function at different stages of pregnancy. Any enterprising young scientist should recognize this as a gold mine from which a lifetime of treasure can be extracted.

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CHAPTER

44

Maternal Brain Adaptations in Pregnancy

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INTRODUCTION

We have selected aspects of this topic that concern the most profound changes in physiology that are a consequence of pregnancy and are regulated by the brain, and the most important changes that prepare for a successful outcome of pregnancy. These changes are driven by actions on the brain of hormones of pregnancy: the steroids estradiol and progesterone and its neurosteroid metabolite, allopregnanolone, and peptide hormones from the placenta (placental lactogen), corpora lutea (relaxin), or anterior pituitary (prolactin) (Figure 44.1). In addition, altered secretion of oxytocin, and actions of oxytocin released in the brain have key roles. Most of the studies referred to in this chapter have been performed on laboratory rats, in which pregnancy lasts for c. 22 days, as such studies allow analysis of mechanisms in detail and depth. Nonetheless, studies on this rodent reveal core principles that can be tested in, and generally applied to, other species, though there are differences in detail.

An important aspect of a successful pregnancy outcome is that adverse programming of the fetus and infant should be minimized, which is an issue of great current interest following the pioneering epidemiological studies of Barker et al.[1] Adverse programming in pregnancy can be a result of exposure to maternal stress hormones or be associated with excessive and imbalanced maternal food intake and obesity, despite appropriate physiological adaptations in the neuroendocrine systems considered here. Postpartum, the quality of maternal care is important for coping with stress in later life, as shown in many studies in the past 30 years,[2] hence understanding the mechanisms that enable rapid and intense expression of maternal care in response to the appearance of the young at birth is providing the basis for understanding variability in its quality. Coping with maternity involves cognitive abilities, and there are changes in performance as a result of pregnancy, with evidence for increased complexity of neural connectivity in the hippocampus.

We begin (see the section Osmoregulation in Pregnancy) by considering the role of the brain in driving the large expansion of blood volume, and reduced blood sodium chloride (NaCl) concentration in pregnancy, a consequence of actions of relaxin on the neural circuitry in the brain hub comprising the lamina terminalis and the hypothalamus, which controls vasopressin and oxytocin neurons and thirst. Relaxin was identified originally as an insulin-like peptide produced predominantly by the corpora lutea of pregnancy that relaxes the pubic ligament but also induces uterine quiescence directly and indirectly via inhibition of oxytocin secretion.[3–6] Its importance, through its actions on the brain, in increased water intake and blood volume expansion in pregnancy was recognized more recently.[7,8] The outcome of these actions of relaxin is resetting of osmoregulatory mechanisms in the hub that controls body fluid homeostasis so the hyponatremic hypervolemia of pregnancy results.

We next review the neural networks in the hypothalamus, brain stem, and reward circuitry that control appetite and metabolism (see the section Food Intake and Metabolism in Pregnancy). It has become clear in the last decade that these networks are complex, involve many groups of neurons organized into several interconnected hubs (in the hypothalamus, brain stem, and midbrain) and utilize many different transmitters and neuromodulators, especially neuropeptides.[9] We have aimed to give a current account of these networks, hubs, and the signaling molecules without pregnancy and indicate

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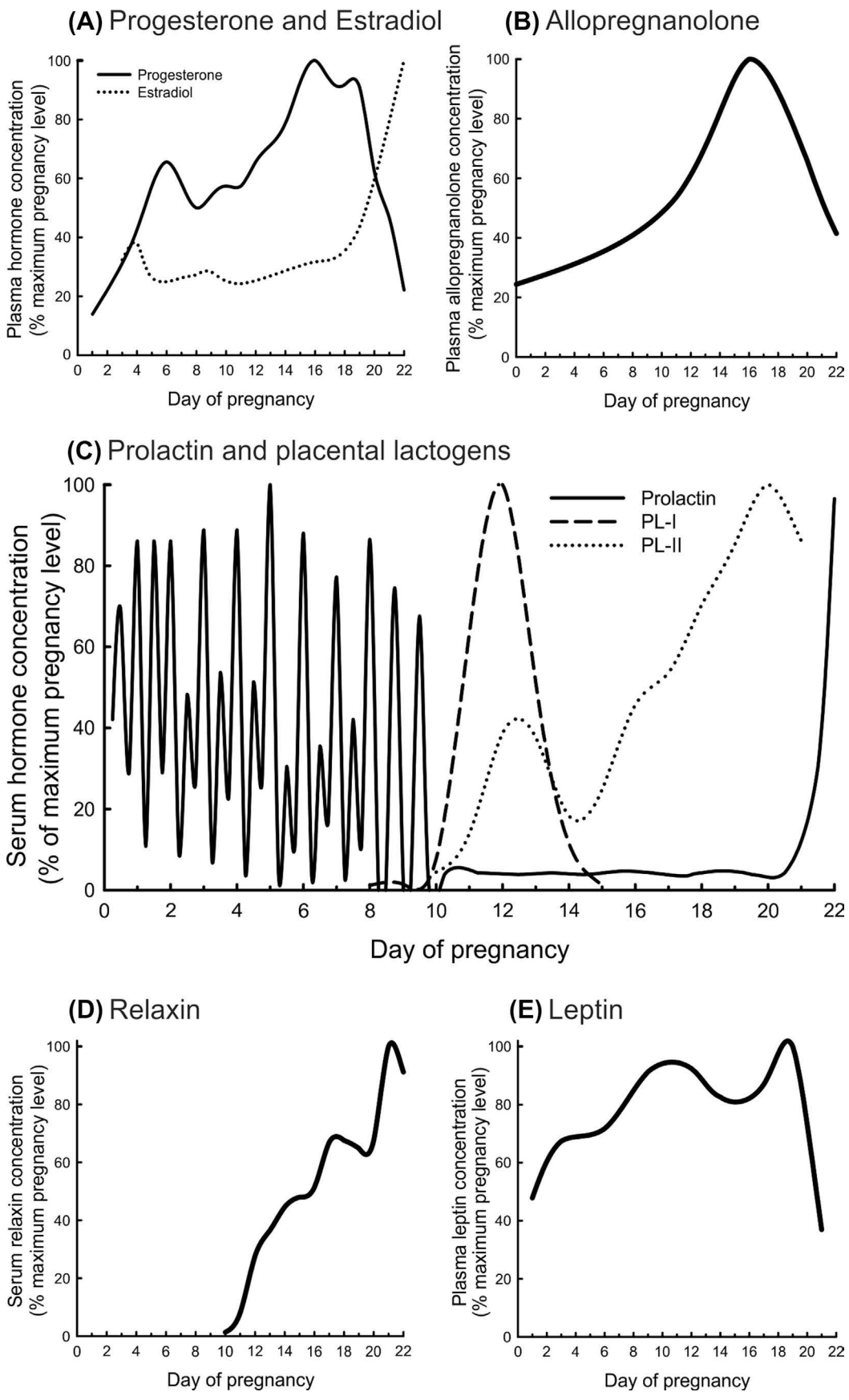

FIGURE 44.1 Hormone secretion profiles during pregnancy in the rat. Circulating concentrations of (A) progesterone and estradiol, (B) allopregnanolone, (C) prolactin and placental lactogen I and II, (D) relaxin, and (E) leptin, across pregnancy in the rat. Hormone concentrations are expressed as a percentage of the maximum levels found in pregnancy. *Sources: Data are derived as follows: progesterone and estradiol;[854] allopregnanolone;[29] prolactin;[424,855] placental lactogen;[856,857] relaxin;[858] and leptin.[859] Reproduced from Brunton & Russell (2010)[860] with permission from Elsevier.*

how they may change in pregnancy. But this is a rapidly advancing field, and while there is a large amount of detail with several dimensions, there are obvious large gaps in present understanding, and these are likely to be filled rapidly. Actions of progesterone have long been considered to be important in driving the increased food intake and energy storage in pregnancy,[10] but with the discovery of leptin,[11] and several other peripheral peptides that signal to the brain and are involved in regulation of appetite and metabolism, attention has focused on changes in these hormones in pregnancy and altered interactions with and adaptations within these central networks. In particular, the importance of resistance to leptin, recognized soon after the discovery of leptin[12] and recently shown to be evident in pregnancy, likely involving prolactin action,[13] and changes in other anorexigens are discussed. Recent evidence for upregulated expression of orexigens (e.g., neuropeptide Y)[14] and uncertainties about downregulated expression of anorexigens in the brain are considered. Hence, this section aims to explain how processing of information about energy supply in the neuronal networks governing regulation of food intake and energy storage may be modified in pregnancy by shifts in the balance between predominance of orexigenic or anorexigenic signals, resulting in increased food intake and energy storage. An important consequence of increased energy storage in pregnancy is that this is available for milk production after birth.

In the prolactin section we discuss changes through pregnancy in the control of prolactin secretion by the brain (see the section The Prolactin System: Preparation for Lactation). With availability of a radioimmunoassay, Friesen and colleagues in 1971[15] found prolactin secretion to be markedly increased in human pregnancy. The main regulation of prolactin secretion without pregnancy, as established in many studies since the early 1970s, is inhibition by dopamine, secreted into the hypothalamo-adenohypophysial portal system by hypothalamic neurons.[16] There are several peptides that are candidate prolactin releasing factors (PRFs), and in early pregnancy increased prolactin secretion (initially pulsatile as identified also in the early 1970s) involves increased PRF actions, then secretion is suppressed by dopamine, but finally increases near the end of pregnancy.[17] Overall, this neuroendocrine system develops changing patterns and levels of prolactin secretion during pregnancy, alongside increasing placental lactogen secretion,[18] appropriate for stimulating appetite, maternal behavior, and lactogenesis.

Responsiveness of the maternal hypothalamo-pituitary-adrenal (HPA) axis to stressors is reduced in pregnancy. The glucocorticoid secreted by this system has powerful effects on metabolism (mobilizing glucose), the cardiovascular system, and immune responses, and on the brain, raising alertness. Attenuation of HPA axis responses in pregnancy, first reported in the 1990s,[19,20] is considered to be beneficial in terms of conserving energy as well as protecting fetuses from adverse programming by excess glucocorticoid (see the section The Hypothalamo-Pituitary-Adrenal Axis). The mechanisms of this adaptation in pregnancy involve activation of an endogenous opioid peptide (EOP) inhibitory mechanism, which has recently been shown to be activated by allopregnanolone,[21] a neurosteroid metabolite of progesterone formed in the brain and in high concentrations near the end of pregnancy, as first reported 20 years ago.[22]

A successful outcome of pregnancy involves functioning oxytocin neuron systems, based in the supraoptic (SON) and paraventricular (PVN) nuclei in the hypothalamus, to support parturition and enable milk transfer after birth (see the section Magnocellular Oxytocin Neuron System in Pregnancy). We discuss their role as a hub for regulation of parturition, the neural inputs that regulate their activity, and how the actions of these inputs are modulated in pregnancy. We detail the autoregulatory mechanisms, inhibitory and excitatory, which are of crucial importance in organizing the coordinated activity of these neurons that leads to secretion of pulses of oxytocin from the posterior pituitary gland, as first described for the milk ejection reflex 40 years ago.[23] A predominant aspect in pregnancy is restraint of oxytocin neurons by several mechanisms that prevent premature activation, including an EOP mechanism in the brain detailed in 1995.[24]

Enhanced cognitive performance in pregnancy is seen in tests of several memory processes, relevant to learning necessary for successful mothering, and focused on the hippocampus, which is a target for several hormones of pregnancy (see the section Cognition during Pregnancy). Testing roles of specific hormones in nonpregnant rats indicates that estrogen actions are important in enhanced cognitive performance in pregnancy, and in underlying changes in long-term potentiation (LTP) at hippocampal synapses that have been revealed in the last few years.[25] Progesterone and allopregnanolone actions are also implicated in hippocampal changes, as is oxytocin, which some 25 years ago was deduced to be important in forming olfactory memory for the young postpartum.[26] Prolactin has recently been shown to play a role in stimulating neurogenesis in the brain in pregnancy,[27] while functional hippocampal synaptic changes in pregnancy were reported in 2006 to be reflected in an increase in number of dendritic spines.[28]

The high levels of progesterone in the brain fall dramatically near the end of pregnancy in the rat, as documented by Concas et al.,[29] and this is an important step permitting the emergence of maternal behaviors, as first reported in 1978,[30] which is stimulated by the high level of prolactin at this time, as first implicated around 50 years ago[31,32] (see the section Maternal Behavior). The

medial preoptic area (mPOA), rostral to the hypothalamus, is the key hub in the neural networks that organize the several elements of maternal behavior, and prolactin acts here, as first reported in 1974[33] and 1990,[34] respectively. Also important is oxytocin, as controversially reported in 1979,[35] but consistent with subsequent evidence for release in the brain and action in the mPOA and at several nodes in the maternal behavior networks.[36]

OSMOREGULATION IN PREGNANCY

Central Control of Decreased Blood Osmolarity and Increased Volume in Pregnancy

Homeostatic mechanisms regulating extracellular fluid osmolality and blood volume are reset in pregnancy. By the end of pregnancy this adaptation increases blood volume by c. 55%, and plasma volume is increased by c. 40%, enabling large increases in blood flow through the placenta and maternal organs supporting the pregnancy.[37,38] The volume increases are accompanied by decreases of c. 4% in plasma [Na^+] and osmolarity.[37] Clearly, there is a large retention of water and NaCl in pregnancy, though the increase in NaCl retention is proportionally less, leading to the hyponatremic hypervolemia of pregnancy.

The brain governs these changes through altered regulation of drinking, and water retention and sodium excretion by the kidneys. Relaxin, a pregnancy hormone, has an essential role in driving this adaptation through its actions on the brain.[39] The altered regulation involves central osmoreceptors, vasopressin (antidiuretic hormone; ADH), and in the rat, oxytocin, secreted from the posterior pituitary gland. These mechanisms interact with regulation of blood volume and sodium ion concentration ([Na^+]) by other mechanisms, in particular actions of angiotensin II and atrial natriuretic peptide (ANP).[40] In the nonpregnant state, stimulation of vasopressin and oxytocin secretion by increased blood osmolarity will lead, through respective actions of vasopressin and oxytocin, to antidiuresis and, in rodents, to natriuresis, hence normalizing osmolarity and blood volume. In pregnancy, the hormone relaxin intercedes to modulate this homeostatic mechanism.

Vasopressin and Oxytocin

Vasopressin and oxytocin are closely related nine–amino acid peptides produced by separate magnocellular neurons in the PVN and SON nuclei, which project their axons to the posterior pituitary gland.[41] The large size of the cell bodies of these neurons reflects the large amount of peptide hormone that they produce; hormone newly synthesized in the cell bodies in the PVN and SON is transported within the axons to the posterior pituitary. Large amounts of these peptide hormones are stored in the axon terminals, and some of the stored hormone is released into the systemic circulation when axon potentials invade the terminals. The rate and pattern of hormone release is determined by the frequency and patterning of action potentials, which is a result of the sum of inhibitory and excitatory synaptic input on the cell bodies, interacting with the intrinsic electrical properties of the magnocellular neurons; typically, vasopressin neurons fire action potentials in phasic bursts, and oxytocin neurons fire continuously and irregularly.[41] However, oxytocin neurons fire in high frequency synchronized bursts during parturition and suckling, which results in the intermittent secretion of pulses of oxytocin to effect parturition and milk ejections (see the section Magnocellular Oxytocin Neuron System in Pregnancy and Chapter 13).

Other neurons in the PVN, but not the SON, that produce either oxytocin or vasopressin have smaller cell bodies. Hence these are designated as parvocellular neurons, which reflects that they produce less peptide than magnocellular neurons as they do not secrete into the systemic circulation. Instead, vasopressin parvocellular PVN (pPVN) neurons, which coproduce corticotropin releasing hormone (CRH), project to the median eminence and release their peptides into the hypothalamo-hypophysial portal system to regulate adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary gland (see the section The Hypothalamo-Pituitary-Adrenal Axis). Parvocellular oxytocin neurons by contrast project their axons within the brain to the brain stem, spinal cord, or to rostral brain regions concerned with emotionality and behaviors, and have important roles in the control of appetite, and maternal and social behaviors (see the section Food Intake and Metabolism in Pregnancy and Maternal Behavior). The central actions of oxytocin released by pPVN neurons may be complemented by the release of oxytocin from the dendrites of magnocellular oxytocin neurons, which certainly has a key role in autoregulating magnocellular oxytocin neurons during parturition and lactation (see the section Magnocellular Oxytocin Neuron System in Pregnancy and Chapter 13).

MAGNOCELLULAR OXYTOCIN AND VASOPRESSIN NEURONS ARE OSMORECEPTORS

Verney demonstrated that osmoreceptors controlling vasopressin secretion and hence water excretion are located in the brain.[42] In the context of regulation of plasma osmolarity and [Na^+], an important factor is the direct sensitivity of the magnocellular neurons to changes in extracellular fluid osmolarity, i.e., these neurons are osmoreceptors.[43,44] This osmosensitivity results in depolarization when extracellular osmolarity increases, and conversely in hyperpolarization when it decreases,[44]

as in pregnancy. However, this direct osmosensitivity requires support from excitatory synaptic input to increase action potentials.[45,46] In particular, the relevant input is from the osmosensitive osmoreceptor complex in the lamina terminalis (Figure 44.2). In addition, glial cells in the magnocellular nuclei release taurine, which enhances tonic inhibition of the neurons by glycine, and this release is reduced with increased extracellular osmolarity;[47] hence, this inhibitory mechanism is expected to be enhanced in pregnancy as osmolarity is decreased. Vasopressin neurons are also stimulated by reductions in blood pressure or volume,[48] and such changes augment responses to hyperosmotic stimulation. This augmentation persists in pregnancy,[38] which may protect the hyponatremic hypervolemia of pregnancy.

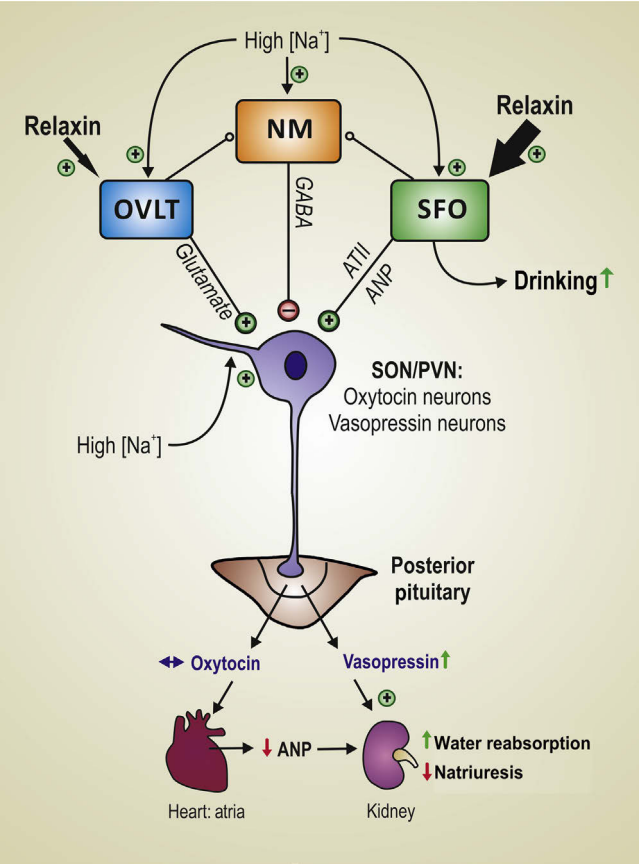

FIGURE 44.2 Mechanisms of hyponatremia and hypervolemia in late pregnancy. The organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) are both strongly interconnected with the nucleus medianus (NM) in the lamina terminalis. Together these structures comprise the AV3V region (the region anterior and ventral to the third ventricle), which plays a key role in regulating fluid and electrolyte balance. Hypernatremia (high [Na^+]) stimulates magnocellular oxytocin and vasopressin neurons in the paraventricular (PVN) and supraoptic (SON) nuclei directly, but also indirectly via osmoreceptive neurons in the OVLT, SFO, and NM. AV3V neurons project to the SON and PVN to regulate the activity of the magnocellular neurons. The projections from the AV3V region involve glutamate (excitatory) and GABA (inhibitory) transmission, atrial natriuretic peptide (ANP), and angiotensin II (ATII). In late pregnancy, relaxin from the corpora lutea acts on subfornical organ (SFO) neurons to stimulate drinking. Relaxin also stimulates magnocellular vasopressin neurons via relaxin receptors in the OVLT and SFO. In early, but not late, pregnancy, relaxin similarly stimulates oxytocin neurons. Increased vasopressin secretion (stimulated by relaxin) acts on the kidney via V2 receptors to increase water retention. This together with increased drinking and reduced natriuresis, as oxytocin secretion is not increased and ANP secretion is decreased, leads to the hyponatremia and hypervolemia of pregnancy.

Lamina Terminalis Complex

The lamina terminalis complex (or AV3V region; the region anterior and ventral to the third ventricle) includes neurons that are osmosensitive. Some of these neurons, in the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), are outside the blood–brain barrier (BBB) and are responsive to circulating angiotensin II, which reflects renal blood flow,[49] and to relaxin.[39] These neurons form an osmoregulatory network, which includes GABAergic and glutamatergic neurons, and neurons that use angiotensin II and ANP as excitatory transmitters.[39,50] They project to the magnocellular oxytocin and vasopressin neurons, and to brain regions that control drinking (Figure 44.2).

The lamina terminalis has an essential role in the regulation of Na^+ excretion as lesion of this brain region strongly reduces excretion of a Na^+ load.[51,52] This has been attributed to failure of ANP release by the brain, or loss of the ANP input from the lamina terminalis to the oxytocin neurons, with resulting reduced oxytocin secretion.[51] ANP mRNA expression in the preoptic area is decreased in late pregnancy,[53] which may contribute to the reduced effectiveness of stimulating the lamina terminalis to increase oxytocin secretion in late pregnant rats.[54]

Vasopressin Neurons and Relaxin

In pregnancy, relaxin secreted by the corpora lutea acts on the brain to reset the osmoregulatory networks and drive water drinking.[49] This is achieved by reduction of the osmotic threshold at which vasopressin secretion is stimulated, without changing the slope relating osmolarity increase to vasopressin secretion.[55] Hence relaxin overrides the inhibitory mechanisms, outlined before, that would otherwise operate. The increased vasopressin secretion induces water retention by renal actions, via V2 receptors,[56] and plasma volume is thereby increased and osmolarity is reduced. This, together with water drinking governed by relaxin and the reduced osmotic threshold in pregnancy,[37,57] accounts for the increased plasma volume. Infusion of relaxin to nonpregnant rats, or central injection of a viral vector to upregulate relaxin expression in the brain, results in increased vasopressin secretion, and drinking, sustained over 3 weeks, and reduces plasma osmolarity as in pregnancy.[58,59]

Relaxin(s) and Receptors in the Brain

Relaxin was originally discovered as a circulating factor in the pregnant guinea pig that softens the pubic ligament, permitting birth of the young in this species.[3] In the pig and rodents it softens the cervix and promotes mammary gland development;[60] in its absence, parturition in rats is prolonged.[61] In addition, relaxin produced by the corpora lutea in early pregnancy has vasodilator actions, which may be important in increasing uterine blood flow as well as being involved in the normal reduction in plasma osmolality and increased cardiac output in pregnancy.[62–65] A proposed role in offsetting preeclampsia in late pregnancy has so far failed to be supported by evidence.[66]

The source of relaxin in pregnancy varies between species, but the corpora lutea are the source in women, pigs, and rats.[60] Relaxin is a peptide, showing some species differences: in women the relaxin secreted in pregnancy is designated human (H) relaxin-2, with biological activity like rat (R1) or porcine (P1) relaxin (in this account all called "relaxin"),[67] and evidently these are the only forms that are secreted into blood. Blood levels increase steadily from the first third of pregnancy[60] (Figure 44.1). Relaxin acts via a leucine-rich guanine nucleotide-binding (G-protein)-coupled receptor, LGR7,[68] now called RXFP1,[69] via stimulation of the G_s-cAMP–protein kinase A pathway.[68,70]

There are two other closely related peptides, products of different genes, relaxin-3 (expressed in the brain, in GABA neurons in the nucleus incertus),[71] and relaxin-1 (expressed in the decidua, placenta, and prostate).[72] The relaxins are members of the insulin superfamily.[60] It has been proposed that relaxin-3 is the evolutionary ancestor of relaxin (e.g., H-2, R1, and P1),[73] but it now seems that the modern vertebrate forms arose from a common ancestral gene.[74] Of interest here, central relaxin-3 administration activates the osmoregulatory network in the brain and increases water intake in rats.[75] Such actions could be through RXFP1.[76] Whether relaxin-3 contributes to increased water drinking and the hyponatremic hypervolemia of pregnancy is not yet clear.

Mechanisms regulating RXFP1 receptor expression in the brain and in the SFO in particular are not known, although progesterone is a positive regulator in the pregnant myometrium,[64] and estradiol treatment decreases relaxin receptor binding in the cerebral cortex.[77]

Relaxin and Water Drinking

Relaxin, tested in nonpregnant rats, acts on subfornical organ (SFO) neurons to stimulate drinking,[49] and the OVLT is more involved in mediating stimulation by relaxin of the magnocellular PVN and SON neurons.[49] In vitro, SFO neurons are electrically activated by relaxin.[49] RXFP1 is quite widely expressed in the brain, and strongly in the SFO, especially its outer part, but not in the OVLT.[78] In pregnancy, increased angiotensin 1A (AT1A) receptor expression in the SFO is expected to increase sensitivity to the dipsogenic action of angiotensin, and hence support water drinking induced by relaxin.[79]

The lamina terminalis structures project widely to interact with defined brain regions concerned with thirst and regulation of drinking, from anterior cingulate cortex and insula to thalamus, midbrain, and hindbrain regions.[44,80] Presumably, relaxin actions on the SFO in pregnancy are transmitted through these projections; whether there are any changes in these regions in pregnancy that increase thirst is not clear.

Oxytocin and Sodium Balance

Oxytocin secretion is stimulated in the rat by increased plasma osmolarity and has natriuretic actions.[81] Magnocellular oxytocin neurons and oxytocin secretion are also stimulated by blood volume expansion.[51,82] The natriuretic action of oxytocin is exerted partly on the kidney,[83] and also via the heart by stimulating ANP release by the atria.[84]

In late pregnancy, oxytocin receptor mRNA level in the heart and kidney, circulating ANP levels, and renal sensitivity to ANP are all reduced.[85–87] Hence the oxytocin-ANP axis that would normally oppose Na^+ retention and blood volume expansion is less active in pregnancy. In addition, in pregnancy, as a result of the reduced osmolarity caused by the stimulation of vasopressin secretion by relaxin, the threshold for osmotic stimulation of secretion of oxytocin is normally not reached; indeed, a hyperosmotic stimulus that does not increase osmolarity above the nonpregnant level is ineffective in pregnant rats.[88] Consequently, under normal conditions in pregnancy, Na^+ excretion is reduced, to an extent that enables the hyponatremia seen in pregnancy. Nonetheless, a sufficiently large hyperosmotic or hypervolemic stimulus in pregnant rats does stimulate oxytocin secretion, as the threshold is exceeded, and the stimulus-response relationship is then similar to that without pregnancy.[89,90]

Relaxin and Oxytocin Neurons

In nonpregnant rats relaxin infusion can stimulate oxytocin neurons, partly via angiotensin II actions.[91,92] However, in late pregnancy relaxin does not stimulate oxytocin secretion, while vasopressin responses to relaxin are retained.[93] Also, ovariectomy on day 15 of pregnancy to remove relaxin (pregnancy maintained with estradiol and progesterone implants) does not restore oxytocin responses to a hyperosmotic challenge, indicating that this lack of response is independent of relaxin actions at this stage.[94] However, initiation of reduced hyperosmotic responses of oxytocin neurons by relaxin in nonpregnant rats, but not increased water drinking or hyponatremia,

requires pregnancy levels of estradiol and progesterone;[95] these experiments were performed in the presence of naloxone to remove any induction by relaxin of inhibition of secretion by endogenous opioid.[61] The mechanisms of this estrogen–progesterone dependent action of relaxin need further study.

Salt Appetite

In rats salt appetite is increased in pregnancy,[37,96] yet with excessive intake, the hyponatremia of pregnancy is maintained.[97] The mechanism of increased salt appetite in pregnancy is not clear. Salt intake is not increased by relaxin in nonpregnant rats,[98] while pregnant mice with inactivation of the serum- and glucocorticoid-inducible kinase (*Sgk1*) gene, important in salt appetite stimulation by mineralocorticoids, show reduced sodium appetite.[99]

Circulating angiotensin II is a signal acting on the brain that can increase salt intake, and angiotensin II produced in the brain is important in stimulating salt appetite, which is usually associated with hypovolemia;[100] this is not the situation in pregnancy as blood volume is increased.

Angiotensin II acts in the lamina terminalis, from where neurons project to regions concerned with salt appetite, though the role of angiotensin II actions in the lamina terminalis, and the SFO in particular, is controversial.[100] Angiotensin II can stimulate and inhibit salt appetite, and the latter action has been shown to be via a central oxytocin action.[100–103] Any role for circulating angiotensin II in increased salt appetite in pregnancy is not clear. However, oxytocin clearly has actions in the brain to reduce food intake and salt appetite.[104–107] Hence, if in pregnancy central release of oxytocin by parvocellular oxytocin neurons is inhibited as well as from magnocellular oxytocin neuron dendrites,[108,109] increased salt intake could be explained, perhaps by unopposed angiotensin II action.

Inhibition of salt appetite is associated with inhibitory action of serotonin from dorsal raphe neurons released in the lateral parabrachial nucleus (lPB), which is a key node in a regulatory network involving brain stem and forebrain structures, including lamina terminalis components.[110] It is not clear whether the lPB is a site of oxytocin action.

Adrenomedullin

This is a potent hypotensive 52–amino acid peptide that has important actions in the periphery in pregnancy (e.g., in implantation and uterine vascular changes).[111,112] It is expressed in reproductive tissues and production, especially by the placenta, and circulating levels increase through pregnancy.[111] Adrenomedullin is also co-expressed in magnocellular oxytocin and vasopressin neurons,[113] and it acts centrally to excite oxytocin neurons.[114,115] Given centrally, adrenomedullin is hypertensive and inhibits water drinking and sodium appetite.[116,117] Brain adrenomedullin is important in mediating stimulation of oxytocin secretion by hypernatremia, and the inhibition of sodium appetite by adrenomedullin involves oxytocin action in the brain.[118] Although changes in pregnancy in the adrenomedullin system in the brain have not been reported, reduced activity and consequent reduced stimulation of oxytocin neurons clearly could have a role in maintaining the hyponatremia of pregnancy.

Sex Steroids

There are several possible sites of action of female sex steroids in the osmoregulatory complex. Estrogen receptor-alpha (ERα) is expressed by neurons in the lamina terminalis (SFO, OVLT, and median preoptic nucleus), and only estrogen receptor-beta (ERβ) is expressed in the magnocellular oxytocin and vasopressin neurons.[119–121] The lamina terminalis neurons expressing ERβ, which project to the magnocellular neurons,[119] respond to osmotic stimulation.[120] Magnocellular oxytocin neurons express G-protein coupled receptor 30 (GPR30),[122] which is a nongenomic estrogen receptor mediating rapid estrogen actions.[123] Only a few neurons in the lamina terminalis express progesterone receptors, but magnocellular neurons lack these.[124]

Summary and Conclusions

In pregnancy relaxin acts on the lamina terminalis and hence the magnocellular oxytocin and vasopressin neurons, together comprising the hub for regulating body fluid osmolarity. Relaxin reduces the threshold for osmotic stimulation of vasopressin neurons, causing water retention and hyponatremia, compounded by increased water drinking, notwithstanding increased sodium appetite. These changes make osmotic and hypervolemic regulation of oxytocin neurons less effective, attenuating any natriuretic action of oxytocin, supported by reduced atrial natriuretic peptide secretion.

Increased oxytocin secretion during parturition and by suckling following parturition, the loss of relaxin at the end of pregnancy, and increased ANP secretion postpartum[86] all contribute to enhanced natriuresis and reversal of the central adaptations in control of osmolarity and blood volume.

Future Perspectives

Mechanisms regulating RXFP1 expression in the brain need to be clarified. The discovery of relaxin-3, its expression in the brain, and that it stimulates water drinking raises questions about possible roles in pregnancy. The explanation for increased salt appetite in pregnancy is not clear, but may involve changes in the neural circuitry controlling salt appetite, or involve angiotensin II actions, or inhibition of central oxytocin or adrenomedullin release.

FOOD INTAKE AND METABOLISM IN PREGNANCY

Increased appetite and adipose storage are important accompaniments of pregnancy. The increase in food intake in women is less than 10% in developed countries and more in less-developed countries.[125] However, there is concern about the impact on pregnancy outcome in preexisting obesity, or obesity that develops during pregnancy.[126] By contrast, in rats food intake is increased in pregnancy by about 60%.[109,127] Increased intake supplies the growing placenta(e), fetus(es), and uterus, and increased maternal metabolism, as well as providing surplus for storage as adipose tissue[128] and preparation of the mammary gland for lactation.[129] Meeting these demands involves resetting of complex neural circuitry involving the hypothalamus, brain stem, and reward circuitry in particular. The mechanisms are not fully understood, but resistance to some normal physiological signals from the periphery that control appetite has been demonstrated, and changes in metabolic control are implicated.[130]

Interest in the regulation of maternal appetite and metabolism in pregnancy has been driven recently by concerns about obesity before and during pregnancy on the mother's health, and about long-term consequences for the health of offspring experiencing an overabundance of nutrients and other food constituents in utero.[131] Increased incidence of obesity in pregnant women, with increased birth weight, over the past 20 years in the United States is a major health concern.[132] This is because obesity in the mother is associated with metabolic disease, including gestational diabetes mellitus, and with programmed metabolic disorder in the offspring,[133,134] as modeled, for example, in sheep,[135,136] mice, and rats.[137–143]

Appetite and Metabolic Regulation

Principles

Calories eaten in excess of current need (i.e., for basal metabolism, exercise, and in pregnancy to supply the fetus(es)) are stored as glycogen in liver (a short-term store), or in muscle (which is only available for use by muscle), or predominantly as adipose tissue (white and brown; a long-term store). Adipose tissue can accommodate by far the largest amounts of stored energy.[144]

The size of this adipose store is a result of the balance (or imbalance) between calories consumed (carbohydrate or fat, with a smaller contribution from protein) and calories used, over a long period of time. In the recent past, the adipose store in human populations was quite stable, and evidently well regulated, but in the last few years the prevalence of obesity (most simply defined as excess body fat, measured by a body mass index [BMI: weight kg/height m^2] >30) has dramatically increased in many prosperous countries (e.g., United States), but not all (e.g., Sweden; World Health Organisation; http://www.who.int/gho/ncd/risk_factors/overweight/en/). In general, this epidemic of obesity is considered to be a result of eating too much and exercising too little; hence the mechanisms that control appetite and metabolism are a focus of much interest. The mechanisms that integrate signals about metabolic status and organize the sensations of hunger and satiety are in the brain, in a neuronal network involving groups of neurons in the hypothalamus and brain stem, in particular, interacting with the mesolimbic dopaminergic reward network in the midbrain and limbic system.[145,146] These networks also exert control over metabolism, especially in brown adipose tissue. A multitude of specific chemical signals in the brain (endocannabinoids (eCBs), monoamines and many neuropeptides) and signals from the periphery (conveyed by the vagus, or circulating peptide hormones) inform the appetite and metabolism controlling networks about metabolic state, which then organize appropriate behavioral and autonomic metabolic regulation (see also Chapter 35).

Understanding of these mechanisms has developed rapidly during the past 20 years, driven especially by the discovery of leptin in 1994[11] and by subsequent discovery, partly through molecular genetics, genome sequencing, and neuropeptidomics, of more peptides with actions on appetite and metabolism.[147] It is clear that most obese people do not have disturbance in function of only a single gene, so the dysregulation of appetite and metabolism in obesity is a result of changes in the settings in the regulatory networks in the brain. These networks are governed by signals that stimulate appetite and energy storage (orexigens) and by opposing signals that restrain appetite and promote metabolism (anorexigens), and by the reward network. Research has been focused on identifying these signaling mechanisms and evaluating their importance.[148] These controls operate over different time domains: the control of eating behavior involves short-term appetite and satiety signals, acting in a context of longer-term regulation of food intake and metabolism.

In pregnancy, there is a clear shift in the operation of the regulatory networks in the brain to favor increased appetite and increased energy storage, while the uterine contents (i.e., fetuses, placentae, membranes), are metabolically highly active. Multiple mechanisms are involved, contrasting with the relaxin-lamina terminalis-vasopressin neuron circuit that is primarily responsible for the hyponatremic hypervolemia of pregnancy (see the section Osmoregulation in Pregnancy). In principle, increased food intake in pregnancy could be a consequence of resistance to anorexigens or increased actions of orexigens; there is evidence for both types of change.

Network Components, Mediators and Modulators

Hypothalamus

More than 70 years ago, the hypothalamus was recognized as containing neural circuits that control appetite; researchers showed that lesions of the lateral hypothalamus led to suppression of eating, and that lesions of the ventromedial nucleus (VMN, or ventromedial hypothalamus) or PVN in the hypothalamus have opposite effects, leading to obesity.[149–152] Hence the medial hypothalamus has been considered a "satiety center" and the lateral hypothalamus a "hunger center" (Figure 44.3).

Neuropeptide Mediators

It was soon established after neuropeptide Y (NPY) was discovered in 1982 that this 36–amino acid neuropeptide is a highly potent orexigen in the brain.[153] The finding that a set of arcuate nucleus neurons express NPY led to focus on this region in appetite regulation (Figure 44.3). NPY neurons co-express agouti-related peptide (AgRP), another orexigen that is longer acting.[154] Pro-opiomelanocortin (POMC) neurons are also clustered in the arcuate nuclei and release α-melanocyte stimulating hormone (α-MSH), an anorectic peptide derivative of POMC, which stimulates metabolism, via melanocortin-4 receptors (MC4), expressed in several brain regions, including the PVN, nucleus tractus solitarii (NTS), lateral hypothalamus, and VMN.[155,156]

AgRP is an antagonist at MC4 receptors, so it blocks α-MSH actions.[157,158] POMC neurons also produce cocaine and amphetamine regulated transcript (CART) in the rat,[159] but not in humans, in which CART is found only in a minority of NPY neurons and is absent in POMC neurons.[160] Overall, CART has anorexigenic and thermogenic actions,[159,161] but applied to the PVN CART is orexigenic.[163]

It is clear that the NPY/AgRP and POMC/CART arcuate nucleus neurons in the base of the hypothalamus, adjacent to the median eminence, where the blood–brain barrier is deficient, are the major first order hypothalamic neurons in the control of appetite and metabolism, positioned to respond to blood-borne metabolic signals.[9] Hence, when stimulated, NPY/AgRP neurons alter activity of second-order neurons in the regulatory network that results in stimulated feeding, and reduced metabolism, in particular neurons in the PVN and dorsomedial hypothalamus (DMH)[163] and, thence, melanin concentrating hormone (MCH) neurons and orexin neurons in the lateral hypothalamus (Figure 44.3).[164,165] These lateral hypothalamic neurons increase feeding[164,166] and affect peripheral metabolism and fat storage via autonomic (sympathetic) outflow.[167] The actions of NPY/AgRP from arcuate neurons on MCH neurons are evidently indirect, via the PVN or DMH.[163,168,169]

Stimulated arcuate nucleus NPY/AgRP neurons release GABA, which inhibits nearby POMC/CART neurons.[170] Conversely, when POMC/CART neurons are stimulated they inhibit feeding, partly through releasing α-MSH to act on MC4 receptors in the PVN to stimulate oxytocin and CRH neurons,[171] and by also inhibiting MCH and orexin neurons they increase metabolism. Actions of α-MSH on MCH neurons are indirect as they do not express MC4 receptors.[169,172]

Fine details of the relative importance of the different components of these networks are being clarified by application of optogenetics (channelrhodopsin-assisted circuit mapping), which involves viral transfection of neurons expressing a specific peptide with the channelrhodopsin gene, which then allows excitation of the cell bodies or axons of these neurons by light pulses from a laser source via an implanted optical fiber. This approach is combined with recording of electrophysiological responses of identified neurons to test whether they are targets for the light-stimulated neurons, and with recording eating behavioral responses.[173] With this combination, it has been shown that stimulation of AgRP neurons (also producing NPY and GABA) in the arcuate nucleus inhibits POMC neurons, but that this connection is not important for acute feeding responses;[173] however, stimulating AgRP inputs to the PVN is important in evoking feeding, and this is mediated by inhibition (via GABA) of oxytocin neurons in the PVN, hence indicating a key role for these neurons in appetite regulation,[173] with implications for the explanation of increased appetite in pregnancy, as discussed following.

Modulators

ENDOCANNABINOIDS

The activity of neurons in the network is modulated by eCBs, generally in such a way that appetite is stimulated; hence a cannabinoid receptor 1 (CB1) antagonist (rimonabant) reduces energy intake, acting on orexigenic and anorexigenic neurons in the hypothalamus, in particular in arcuate neurons decreasing NPY production and increasing α-MSH and CART production.[174] To date, roles in appetite and metabolism regulation in pregnancy have not been explored.

SEROTONIN

Serotonin (5-HT) depletion or inactivation of specific serotonin receptor subtypes leads to obesity. Consequently, drugs that increase serotonin availability (e.g., D-fenfluramine or sibutramine) and a serotonin 2_C-receptor (5-HT_{2C}R) agonist (lorcaserin) have antiobesity actions.[147] The 5-HT_{2C}R mediates the stimulatory actions of serotonin on POMC neurons, independently of leptin, while the 5-HT_{1B}R mediates the inhibitory actions on AgRP neurons; the actions on POMC neurons lead to anorectic effects via MC4R in the PVN.[147] Any

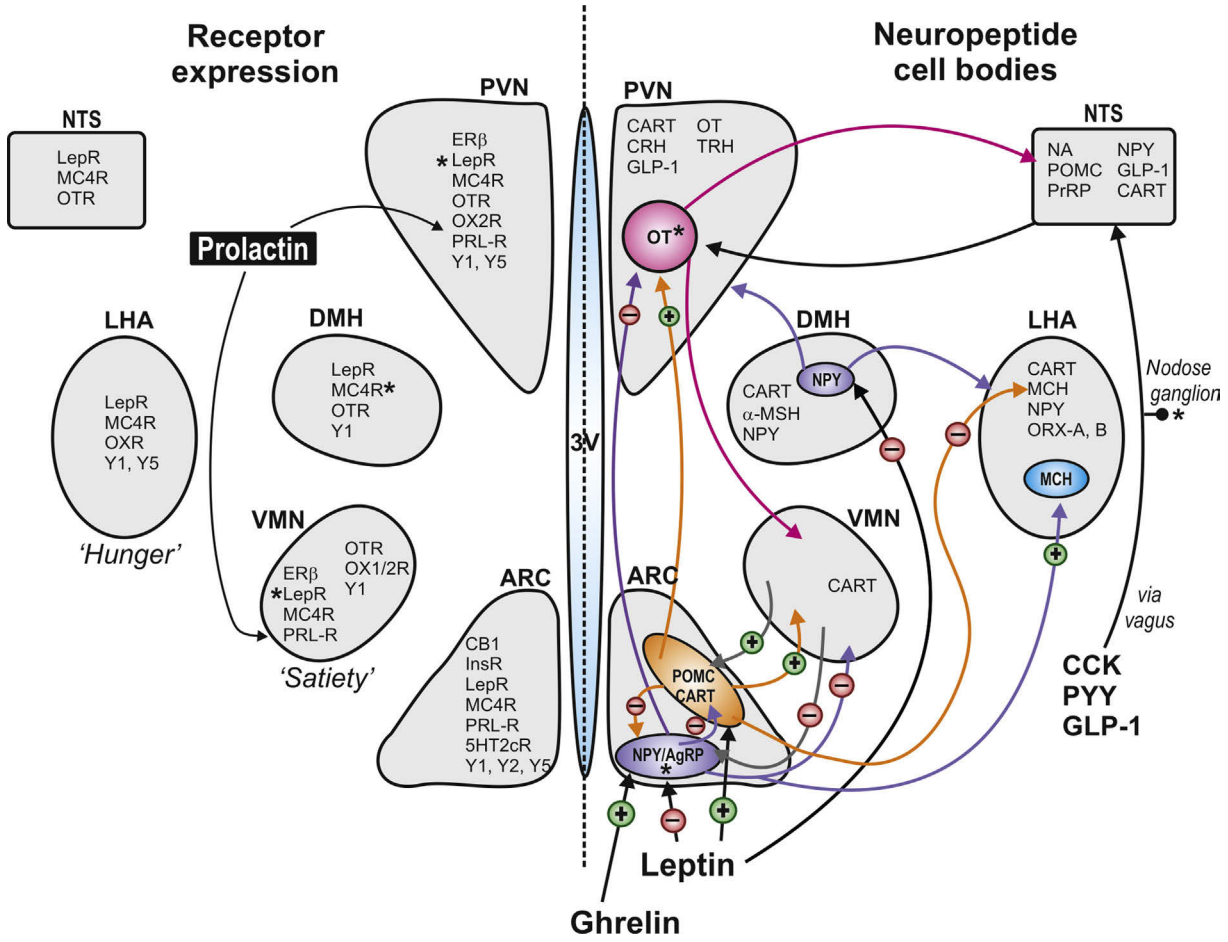

FIGURE 44.3 Hypothalamic neuropeptides and networks regulating appetite and metabolism. The arcuate nucleus (ARC) contains NPY/AgRP neurons that increase food intake and energy storage via inhibition of adjacent POMC neurons (producing anorexigens-α-MSH and CART) and anorexigenic neurons in the PVN (OT, CRH) and via antagonism of α-MSH action on MC4 receptors that mediate inhibition of MCH neurons in the LHA ("hunger center"), with actions in the VMN ("satiety center"). POMC/CART neurons in the arcuate nucleus decrease food intake and increase metabolism by inhibiting NPY/AgRP neurons, stimulating oxytocin neurons, and inhibiting MCH neurons in the LHA. Arcuate NPY/AgRP neurons are inhibited by leptin and stimulated by ghrelin. Leptin stimulates POMC/CART neurons. Leptin can act at all sites in the network (note leptin receptor distribution, LepR). There are reciprocal connections between PVN anorexigenic oxytocin neurons and the nucleus tractus solitarii (NTS). The NTS processes input via the vagus from gut hormones signaling satiety (CCK, GLP-1, PYY). Noradrenaline, α-MSH (from POMC neurons), PrRP, and GLP-1 are transmitters in the NTS to PVN pathway. In mid- to late pregnancy several changes that alter the network balance in favor of increased food intake and energy storage have been identified to date (*): (1) NPY/AgRP neurons are more active, and evidently leptin resistant as leptin levels are high in pregnancy; (2) LepR expression in the hypothalamus (but not arcuate nucleus) is reduced; (3) leptin resistance (with reduced LepR expression and signaling) develops in the PVN and VMN (induced by prolactin/placental lactogen), and vagal nodose ganglion; (4) activity of PVN oxytocin neurons may be suppressed—they develop resistance to CCK action, and central resistance to anorectic OT actions develops; (5) central resistance to α-MSH develops. Symbols: arrows indicate targets; +: represents excitatory action; –: represents inhibitory action. Regions: 3V, 3rd ventricle; ARC, arcuate nucleus; DMH, dorsomedial nucleus; LHA, lateral hypothalamic area; NTS, nucleus tractus solitarii; PVN, paraventricular nucleus; VMN, ventromedial nucleus. Left: R, Receptors: 5HT2cR, serotonin 2c; CB1R, cannabinoid 1; ERβ, estrogen; InsR, insulin; LepR, leptin; MC4R, melanocortin; PRL-R, prolactin; OTR, oxytocin; OXR, OX1R, OX2R, orexin; Y1, Y5, neuropeptide Y. Right, Neuropeptides: α-MSH, melanocyte stimulating hormone; AgRP, agouti-related peptide; CART, cocaine and amphetamine regulated transcript; CRH, corticotropin releasing hormone; GLP-1, glucagon-like peptide; MCH, melanocyte concentrating hormone; NPY, neuropeptide Y; ORX-A, B, orexins; OT, oxytocin; POMC, pro-opiomelanocortin; PrRP, prolactin releasing peptide; TRH, thyrotropin releasing hormone. Monoamine- NA, noradrenaline. Blood-borne: CCK, cholecystokinin; PYY, peptide YY; GLP-1: glucagon-like peptide. *Source: Diagram adapted from Parker & Bloom.*[163]

role for changes in these central serotonin mechanisms regulating appetite and metabolism in pregnancy has evidently not been investigated.

Adipose Signaling to the Networks Regulating Appetite and Metabolism

Adipokines

Adipokines are peptides produced by adipose tissue with regulatory actions, some of which act on the brain. The adipokines include leptin, adiponectin, resistin, visfatin (from visceral fat), retinol-binding protein 4 (which induces insulin resistance), and vaspin (found in visceral fat and has insulin-sensitizing actions).[175]

Leptin

Leptin is produced by adipose tissue in amounts that reflect fat mass, hence its concentration in the circulation reflects fat stores.[12] It is distinguished by its role in inhibiting appetite and promoting metabolism through actions in the brain, on the network outlined earlier (Figure 44.3).

LEPTIN RECEPTORS

There are two types of leptin receptor in brain, the long form (LEPRb), which mediates the actions of leptin, and the short form (LEPRa), expressed in the choroid plexus and mediating transport of leptin across the BBB into brain.[176] LEPRb is expressed in arcuate POMC and AgRP/NPY neurons, and leptin stimulates the former and inhibits the latter.[177] Hence leptin reduces eating and stimulates metabolism by actions on the neurons at the portal of the network regulating appetite and metabolism. However, there are also neurons in the PVN, VMN, lateral hypothalamus, dorsomedial hypothalamus, and NTS that express LEPRb, and these contribute importantly to the actions of leptin on appetite and metabolism.[178–182] Leptin excites most neurons in the PVN, which has an inhibitory role in appetite regulation.[183] Lack of leptin or LEPRb results in hyperphagia and extreme obesity.[184] Leptin and insulin interact on POMC neurons to regulate glucose homeostasis.[185,186]

Leptin also acts on other central nervous system (CNS) sites to regulate energy balance. For example, leptin can act directly on dorsomedial hypothalamic neurons to stimulate brown adipose tissue (BAT) thermogenesis, by activating sympathetic outflow;[179] and in vitro it acts directly on vagal ganglion afferent neurons (through which cholecystokinin [CCK], for example, signals to terminate a meal),[187,188] and in the NTS. Neurons in the reward circuitry (discussed following) also express leptin receptors, and leptin regulates effort-based feeding.[189]

Insulin Signaling to the Brain

Arcuate NPY/AgRP and POMC/CART neurons express insulin receptors (InsR) as well as leptin receptors.[190] Insulin, secreted by pancreatic islet β-cells, signals plentiful energy like leptin, and it has similar central actions to leptin: it reduces appetite and stimulates metabolism[191] by increasing activity of sympathetic outflow to BAT.[192] These actions contrast with its peripheral actions to drive glucose into tissues for use or storage, as fat or glycogen.

Leptin Resistance

Leptin resistance is the state of increased circulating levels of leptin and decreased inhibitory actions on appetite and decreased stimulatory actions on adipose tissue metabolism. Typically this is the situation in obesity, in which circulating leptin levels are increased as a result of the increased body fat mass.[193] Such leptin resistance essentially describes the failure of leptin to control or reverse obesity. An issue is whether leptin resistance leads to obesity, or vice versa.[194] A common laboratory model to study leptin resistance is diet-induced obesity (DIO) in rats or mice susceptible to obesity if fed a high fat diet.[195] The model has complex elements (e.g., low-grade inflammation, including of the hypothalamus),[194,196] that make detailed comparisons with changes in pregnancy difficult, though obesity models have given leads.[197,198]

Leptin (and insulin) resistance in DIO involves activation of a protein kinase (inhibitor of nuclear factor kappa-B kinase [IKKβ]) and endoplasmic reticulum stress while interleukin-6, an anti-inflammatory cytokine released from exercising muscle, reduces appetite and reduces hypothalamic inflammation in DIO.[196] These issues are presently unexplored in the maternal hypothalamus in pregnancy.

Leptin Resistance in Pregnancy in Women

Pregnancy is a state in which appetite is increased, fat is stored (but mobilized near the end of pregnancy), and circulating leptin levels are increased.[199,200] It is likely that the placenta contributes significantly to circulating leptin levels as plasma concentrations fall rapidly after birth.[199]

In women, these findings have been interpreted to indicate physiological leptin resistance in pregnancy.[201,202] However, in healthy women studied before and during pregnancy, no change in the correlation between leptin level and body fat store is found. Rather, fat store size in pregnancy is more steeply correlated with the homeostasis model assessment of insulin resistance (HOMA-IR), indicating insulin resistance in late pregnancy,[199] which would make

glucose more readily available for the fetus. Nonetheless, more direct indicators of leptin actions in cerebrospinal fluid (CSF) samples, taken just before term in healthy women and from healthy nonpregnant women, have shown signs of reduced entry of leptin into the brain (reduced ratio of CSF: plasma levels) despite increased plasma concentrations. Importantly, CSF levels of AgRP are higher in pregnancy, with no change in POMC levels, hence the AgRP:POMC ratio is increased. These findings indicate reduced leptin transfer across the BBB, perhaps related to increased circulating levels of the soluble form of leptin receptor (LEPRe; OBRe), and reduced inhibition of AgRP release (but unaltered action on POMC), together consistent with increased appetite (via AgRP/NPY release in the brain) and increased fat storage (via inhibition of α-MSH action by AgRP).[200] LEPRe is produced by the placenta and acts to bind circulating leptin, reducing its availability to neural tissues.[203]

Leptin Resistance in Pregnant Rodents

Details of mechanisms of leptin resistance in pregnancy have been studied in detail in rats, in particular, and in mice. Food intake in rats increases early in pregnancy, and this is generally found before leptin levels rise, i.e., before leptin resistance develops, and hyperphagia is sustained through pregnancy despite increasing leptin levels.[204,205] However, as 32 different candidate genes have been identified as contributing to human obesity,[147] changes in pregnancy in the activity of a similar large number of genes in the brain regulating appetite and metabolism is possible.

Early Pregnancy in Rats

Pseudopregnant rats (i.e., rats with a prolonged luteal phase consequent on sterile mating) have modestly increased estrogen, high progesterone, and high but pulsatile prolactin levels, and rapidly become hyperphagic and store fat, but show no changes in leptin levels or in arcuate nucleus NPY, AgRP, or POMC mRNA expression, so are not considered leptin resistant.[130,198,205] Moreover, pseudopregnant rats on day 9 show unaltered hypothalamic intracellular signaling responses (see following) to intravenous leptin.[205]

Hence hyperphagia in early pregnancy is due to early pregnancy hormones. But the mechanisms are not clear—only subsequently are leptin resistance and hypothalamic neuron changes important. By contrast with pulsatile prolactin secretion in early or pseudopregnancy, in the second half of pregnancy placental lactogen is continually produced (Figure 44.1), and this pattern, as modeled by continuous intracerebroventricular (i.c.v.) prolactin infusion with progesterone supplementation, induces leptin resistance.[130]

Estradiol, Food Intake and Metabolism

It is well established that energy intake decreases during the ovarian cycle when estrogen levels peak. Moreover ovariectomy increases food intake and fat deposition in several species, and estrogen replacement prevents this.[206–208] Hence, estradiol is an anorexigen and increases energy expenditure, by acting centrally in the appetite and metabolism regulatory network via first-order arcuate neurons, inhibiting NPY/AgRP neurons and stimulating POMC neurons[209–211] and via second-order neurons in the medial hypothalamus (whether in the PVN or VMN is controversial, although tamoxifen, an estrogen receptor modulator, has anorectic actions via inhibitory actions on fatty acid synthase in the VMN),[212] lateral hypothalamus, and NTS.[206,207,213–216] Estradiol given to ovariectomized rats blocks the orexigenic effect of administration of ghrelin (an orexigenic peptide hormone secreted by an empty stomach, production of which is evidently greater in pregnancy),[217] enhances anorexigenic effects of CCK and CCK-induced stimulation of neurons in the PVN and NTS,[207,218] enhances actions of leptin on food intake,[219] and increases LEPRb mRNA expression in the hypothalamus,[220] without changing plasma or CSF leptin levels.[221] In addition, estrogen and progesterone do not alter leptin transfer across the choroid plexus.[205] As central actions of estrogens without pregnancy are evidently anorexigenic, they are unlikely to increase appetite and energy storage in pregnancy. However, estrogens have essential actions by increasing PR expression in the brain,[222] hence enabling orexigenic actions of progesterone, as discussed next.

Progesterone, Food Intake and Metabolism

A stimulatory effect of progesterone on body weight and fat storage was shown long ago in female mice and rats,[10,223] and in women weight gain in pregnancy correlates with progesterone levels.[224] Progesterone does not affect food intake in the absence of estrogen,[206] but food intake in women during the ovarian cycle is greatest in the luteal phase, when progesterone levels are high.[208] Evidently, progesterone overcomes the inhibitory effects of estradiol.[225]

Chronic progesterone administration in ovary-intact rats increases (for the first 2 weeks) food intake and fat storage while reducing thermogenesis, without changing plasma or CSF leptin levels for up to 30 days.[226] Similarly, in early pregnancy such effects of progesterone do not lead to leptin resistance as an orexigenic mechanism.[130] However, chronic estrogen and progesterone treatment in ovariectomized rhesus monkeys blocks short-term anorexic actions of peptide YY ($PYY_{(3\text{-}36)}$; a 36–amino acid anorectic peptide, related to NPY, that is secreted by the distal intestine after a meal

and acts on Y2 inhibitory autoreceptors, which arcuate NPY neurons express, through which PYY/$PYY_{(3-36)}$ can suppress further feeding after a meal).[227,228,229]

The actions of progesterone on food intake and fat deposition may involve the progesterone receptors that are expressed in VMN and arcuate neurons.[124]

Allopregnanolone, Food Intake and Metabolism

Levels of this neuroactive steroid metabolite of progesterone are high in late pregnancy,[29] and given acutely it stimulates food intake in male rats.[230] Although allopregnanolone is an allosteric modifier at $GABA_A$ receptors, its stimulatory action on food intake in male rats is evidently not mediated by these receptors.[230] However, in female rats stimulation of feeding by allopregnanolone (an effect that is maximal at diestrus; i.e., with low estrogen and progesterone levels), is attenuated by a $GABA_A$-receptor antagonist.[231] Hence, high levels of allopregnanolone in pregnancy may contribute to increased food intake, perhaps via actions on GABA receptors on arcuate POMC neurons and/or on PVN and SON oxytocin neurons: reinforcing inhibition by GABA from NPY/AgRP neurons. This remains to be tested.

Mid-to-Late Pregnancy in Rodents

Changes in Leptin Availability

As in women, leptin levels in blood increase in pregnant rats and mice after the first week of pregnancy, as a result of the increased fat mass[13,198] and a possible contribution from the placentae.[203,232,233] However, more circulating leptin is bound to circulating LEPRe, which seems especially important in mice.[234] Leptin bound to LEPRe is less effective at binding to LEPRb, and in activating postreceptor signaling, i.e., STAT-3 tyrosine phosphorylation.[235] Leptin resistance in pregnancy (from day 13) involves reduced entry into brain through the BBB.[205] This may be a result of action of prolactin on leptin transport across the choroid plexus.[205]

LEPTIN RECEPTORS

Leprb mRNA expression in the hypothalamus is reduced[232,233] or not changed in late pregnant rats or mice,[204,236,237] although reduced hypothalamic LEPRb protein level has been reported on gestation day 18 but not 13.[205] LEPRb expression in the arcuate nucleus is not reduced in pregnancy.[238]

Leptin Resistance in Pregnancy: Functional Evidence

Reduced effects of the high circulating levels of leptin with respect to its central appetite suppressing actions and enhancing actions on adipose tissue metabolism is a feature of the second half of pregnancy in rats and mice. This can support an anticipatory increase in food intake and a positive energy balance.[13,198,239,240] Leptin resistance is revealed by finding that i.c.v. or systemic infusion of leptin fails to reduce food intake in mid-pregnant rats.[13,205,241] Consequently, in rats near the end of pregnancy, *Npy* mRNA expression in the arcuate nucleus is increased, despite increased circulating leptin levels.[14,205,242] A similar pattern in arcuate *Npy* mRNA levels has been reported in pregnant mice,[237] though other studies suggest it is unaltered.[236] In rats, *Agrp* mRNA level has been found to be unaltered[242] or increased, as in mice,[205,236] and *Pomc* mRNA expression has been found to be reduced[205] or not significantly reduced,[242] as in mice.[236] However, the level of *Pomc* mRNA in the arcuate, or the number of arcuate *Pomc* mRNA expressing neurons in the caudal part of the arcuate, is increased in the last week of pregnancy.[243,244] But the arcuate shows increased density of fibers containing β-endorphin, an opioid peptide product of POMC, and α-MSH density has not been assessed in this way.[244] In another species (Brandt's vole) there is no change in hypothalamic *Leprb*, *Npy*, *Agrp* or *Cart* mRNA levels, but a decrease in *Pomc* mRNA is found.[245]

Leptin Signaling and Resistance

Intracellular Mechanisms of Leptin Action

Leptin acts on arcuate neurons to reduce *Npy*/*Agrp* gene expression and to increase *Pomc*/*Cart* expression.[246] Post-LEPRb signaling mechanisms for leptin actions on neurons first involve Janus family kinases (Jak2) and phosphorylation of specific tyrosines in the receptor-Jak2 complex (Figure 44.4), of which: Tyr_{985} on the receptor recruits SH2-containing tyrosine-phosphatase (SHP-2), which activates extracellular signal-regulated kinase (ERK); receptor Tyr_{1138} recruits signal transducer and activator of transcription 3 (STAT3). pSTAT3 is a transcription factor, but also induces suppressor of cytokine signaling 3 (SOCS3), the negative feedback regulator of LEPRb-STAT3 signaling,[247] and positive regulators for leptin actions, e.g., POMC;[248] Tyr_{1077} phosphorylation recruits STAT5[249] (Figure 44.4). pSTAT3 has the major role in mediating leptin actions.[250] The Tyr_{1138} -to- STAT3 path increases *Pomc* gene expression in arcuate neurons[247] and inhibits *Agrp* gene transcription.[251] Leptin also decreases activity of MCH neurons in the lateral hypothalamus, although leptin action is through inputs (perhaps from the arcuate nucleus) rather than directly on the MCH neurons.[252,253] In contrast, orexin neurons in the lateral hypothalamus receive inhibitory input from local neurons expressing LEPRb.[253] Hence leptin signaling in these different neurons, or their inputs, mediates inhibition of appetite and stimulation of metabolism. However, pSTAT3 actions on POMC expression require removal, by phosphorylation,

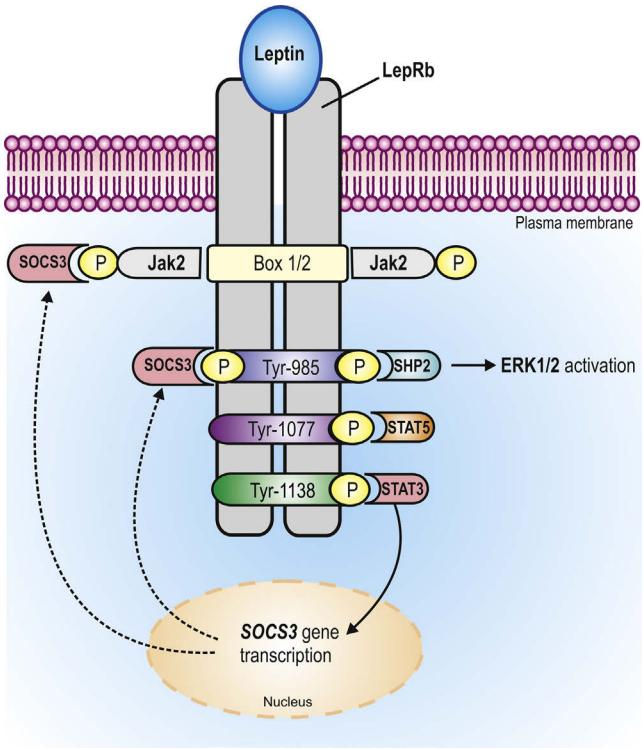

FIGURE 44.4 Intracellular leptin receptor signaling. Binding of leptin to the long form of the leptin receptor (LepRb) results in activation of Janus kinase (Jak2) at the Box 1/2 motif on the LepRb. Activated Jak2 auto-phosphorylates and also phosphorylates (P) tyrosine (Tyr) residues on the LepRb at positions 985, 1077, and 1138. Phosphorylated Tyr-985 is a docking site for SH2 domain-containing phosphatase (SHP2). Phosphorylated Tyr-1077 is a docking site for signal transduction and activation of transcription 5 (STAT5), and phosphorylated Tyr-1138 is a docking site for signal transduction and activation of transcription 3 (STAT3). Activated SHP2 activates the extracellular-regulated kinase 1/2 (ERK1/2) signaling pathway that results in increased EGR-1 gene transcription. Activated STAT3 translocates to the nucleus and induces the expression of suppressor of cytokine signaling 3 (SOCS3). SOCS3 negatively feeds back to inhibit leptin signaling by binding to phosphorylated Tyr-985 and phosphorylated Jak2. Functional leptin resistance in pregnancy may be associated with reduced pSTAT3 formation in response to leptin in neurons that mediate anorectic actions of leptin; this may not simply involve increased SOCS3 induction.

of inhibition by forkhead box protein O1 (FOXO1). Indeed, sustained activation of STAT3 can, by increasing SOCS3, lead to leptin resistance in POMC neurons.[247,254]

In addition, leptin receptor phosphorylation activates, via phosphatidylinositol (3,4,5)-triphosphate (PIP3), phosphatidylinositol 3-kinase (PI3-K),[254] hence converging leptin signaling with insulin signaling (see following). This pathway has been suggested to be important in the regulation of NPY expression by leptin.[198] FOXO1 is one target of p-protein kinase B (a serine/threonine specific kinase, p-Akt), and FOXO1 phosphorylation enables pSTAT3 stimulation of POMC gene expression, suppression of AgRP expression, and stimulation of PVN neurons.[255] Whether there are changes in pregnancy in FOXO1 mechanisms has not been reported.

Insulin Signaling

Occupation of the insulin receptor by insulin activates the insulin receptor substrate (IRS) protein to PI3′-K path by Jak2 and leads to altered membrane potential in appetite regulating neurons; in POMC neurons leptin and insulin activate this pathway but have opposite actions on electrical activity (leptin depolarizes; insulin hyperpolarizes).[256] In AgRP neurons insulin also activates this path, but actions of leptin on POMC neurons lead, via synaptic connections, to inhibition of this particular path in AgRP neurons,[257] perhaps by GABA, which POMC neurons also produce.[258]

Intracellular Mechanisms of Leptin Resistance

From obesity models leptin resistance was proposed to follow exposure of neurons to high levels of leptin that cause continual activation of pSTAT3 and the inhibitor SOCS3.[197] Studies of leptin resistance in pregnancy have tested this mechanism of leptin resistance in different components of the appetite and metabolism regulating networks. There are major differences from obesity models.

At the level of the whole hypothalamus, by contrast with actions in virgins, peripheral leptin injection in rats and mice fails, from gestation day 13, to increase hypothalamic pSTAT or pAkt (a proto-oncogene product: a serine–threonine protein kinase related to PKC) content (reflecting reduced activity of the PI3′-K, -Akt cascade).[205] I.c.v. leptin injection from day 13 also fails to increase hypothalamic pSTAT3 and SOCS3, or pAkt (excepting day 13), although basal SOCS3 levels in pregnancy are greater than in nonpregnant rats.[205] Measurements on micropunch samples indicate that leptin does not increase pSTAT3 in the arcuate nucleus, but induction is decreased in the VMN.[13] However, immunohistochemical studies revealed that arcuate α-MSH (POMC) neurons do not develop leptin resistance in pregnant rats or mice as they show normal pSTAT3 responses to leptin; instead, in late pregnant rats and mice, VMN neurons do not show a pSTAT3 response to leptin, and in rats VMN neurons show reduced LEPRb mRNA expression.[238,240,242] Moreover, the anorectic actions of α-MSH are lost, so the intact pSTAT3 responses of arcuate POMC neurons to leptin in pregnancy are nonetheless ineffectual.[240,242] Evidently, leptin resistance in pregnancy of α-MSH mechanisms resides in targets for α-MSH and not in POMC neurons. These findings contrast with obesity models in which arcuate POMC neurons develop leptin resistance and, tested with MCR3/4 receptor agonist or antagonist, α-MSH signaling in the appetite and metabolism regulating circuitry is maintained or even enhanced.[197]

The loss of anorectic action of α-MSH in pregnancy contrasts with maintained eating responses to i.c.v. NPY or orexin in late pregnant rats, despite loss of oxytocin and HPA axis responses.[259,260]

Prolactin and Leptin Resistance

Prolactin and leptin are members of the cytokine class superfamily of peptides and have similar receptor-mediated intracellular signaling mechanisms.[261,262] However, prolactin has opposite effects to leptin on food intake: infusion of prolactin into the PVN in virgin rats increases food intake,[263] and i.c.v. infusion for 10 days increases food intake and prevents acute anorectic effects of i.c.v. leptin.[264] This effect of prolactin is associated with reduced Fos (an immediate early gene product, marking recent neuronal activation) and pSTAT3 induction by leptin in the PVN and VMN, with no effects of prolactin in the arcuate (Figure 44.3).[264]

Rats in pseudopregnancy (extended by progesterone implants and given chronic i.c.v. prolactin to mimic continuous high levels of placental lactogen seen in pregnancy) (Figure 44.1), show resistance to suppression of food intake by leptin, as in pregnancy.[130] The mechanisms of this action of prolactin are not clear, although prolactin targets many neuron types in the brain, in which it activates pSTAT5.[265] Perhaps relevant is that oxytocin has anorectic actions, oxytocin neurons are excited by leptin[266] and express the long-form prolactin receptor (PRL-R_L), and that prolactin has an opposite action, inhibiting the electrical activity of magnocellular SON oxytocin neurons (although it stimulates Fos and oxytocin gene expression in these neurons).[267] If leptin resistance develops in specific neurons as a result of chronic exposure to prolactin, it seems likely that this will involve interference between prolactin and leptin postreceptor signaling mechanisms. In the PVN the same neurons express LEPRb and PRL-R, and leptin resistance is seen in lactation, as leptin does not induce pSTAT3.[268] However, direct testing of cross-resistance in a cell line expressing both receptors shows that leptin or prolactin overexposure induced homotypic resistance, examining STAT3 or STAT5 signaling, respectively, but cross-resistance was not seen. This finding leads to the suggestion that direct stimulation of PVN NPY neurons, rather than SOCS3 induction, may counteract inhibition by leptin.[268]

Adiponectin in Pregnancy

Adiponectin is insulin-sensitizing, anti-inflammatory, and anti-atherogenic.[175] High leptin levels and low adiponectin levels are associated with gestational diabetes mellitus.[175] Insulin resistance develops[270] and leptin levels increase,[271] while circulating adiponectin levels decrease in late pregnancy, as adipose tissue expands.[272,273] In obese individuals adipose tissue shows markers of chronic inflammation, which persist into pregnancy in pregravid obese women.[274] In women, changes in the adipose transcriptome, including increased expression of inflammatory markers, are evident in early pregnancy, long before insulin resistance in late pregnancy.[275] Mice in late pregnancy show insulin resistance and markers of low-grade inflammation in adipose tissue.[276]

Direct central actions of adiponectin are indicated by expression of adiponectin receptors in arcuate NPY and POMC neurons.[277] Given centrally in mice, adiponectin stimulates energy expenditure and decreases body weight.[278] However, there is evidence for stimulatory effects of adiponectin on food intake: chronic i.c.v. infusion in rats increases energy intake (but increases expenditure by fat),[279] and given systemically, adiponectin increases food intake and decreases energy expenditure in fasted mice, while mice deficient in adiponectin show decreased food intake.[280] Also, adiponectin inhibits magnocellular and parvocellular (those projecting to the NTS) PVN oxytocin neurons, which may increase appetite (see following).[281,282] However, such actions of adiponectin in the increased appetite of pregnancy are unlikely as circulating levels of adiponectin are low in late pregnancy.[283] Nonetheless, it is possible that the reduced level of adiponectin in pregnancy leads to central insulin resistance, which would diminish central anorexigenic actions of insulin.[191]

Oxytocin and Appetite

It has been known for over 25 years that CCK stimulates oxytocin secretion via the vagus, and for 20 years that accompanying decreased food intake is a result of oxytocin release into the brain, specifically from PVN neurons projecting to the dorsal vagal complex (Figure 44.3).[284–286] Hence, oxytocin given into the brain has anorectic actions.[285,287] Oxytocin has rather subtle actions in the brain that reduce appetite, in particular in terminating a meal;[288] in its absence or with an oxytocin receptor (OTR) antagonist, appetite for carbohydrate is increased.[289–291] In rats, oxytocin neurons in the SON are activated during a meal,[292] as shown by Fos expression, and in mice PVN oxytocin neurons are activated at the end of a liquid meal.[291] The importance of PVN oxytocin neurons in food intake regulation is indicated by studies on mice heterozygous for inactivation of the single-minded 1 (*SIM1*) gene (essential for normal PVN development), which show hyperphagic obesity that is rescued by central oxytocin administration, and mice homozygous for conditional postnatal *SIM1* knockout as adults develop hyperphagic obesity and have very low oxytocin mRNA levels in the PVN.[152,293,294] Moreover, mice with oxytocin neurons overexpressing synaptotagmin-4 (which negatively regulates oxytocin exocytosis)

are obese.[295] However, specific deletion of oxytocin neurons in adult mice does not affect food intake or energy expenditure on a regular diet but reduces expenditure on a high fat diet.[296]

Oxytocin circulating at physiological concentrations cannot enter the brain, because entry is effectively prevented by the blood–brain barrier,[297] and it has not been shown to act directly on circumventricular organs, which evidently lack OTRs, although *Oxtr* mRNA is expressed in the lamina terminalis.[298–300] However, oxytocin is released within the brain by axon terminals of PVN parvocellular neurons,[301] and by dendrites of SON and PVN magnocellular neurons, from which it may diffuse to nearby nuclei containing neurons that express oxytocin receptors.[108]

In relation to appetite regulation, oxytocin receptors are expressed by neurons in the VMN,[298,299,302] and the NTS.[303] Estradiol alters architecture of dendrites extending from the VMN, enhancing oxytocin innervation, while progesterone has opposite action.[304] Any such change in pregnancy due to high progesterone level would be expected to reduce inhibition of appetite by oxytocin.[304]

Arcuate POMC (α-MSH) and Oxytocin Neurons

Oxytocin plays a key role in mediating the anorectic actions of α-MSH as they are blocked by central administration of an oxytocin antagonist.[305] Arcuate POMC (β-endorphin/α-MSH) neurons project to the dorsal SON, where oxytocin neurons are predominant[244,306] and to the non-magnocellular division of the PVN (Figure 44.3).[307]

α-MSH acts via MC4R on magnocellular oxytocin neurons to inhibit their firing and consequently oxytocin secretion from the posterior pituitary. The action on firing rate is mediated by locally produced eCBs.[308,309] Remarkably, at the same time, α-MSH acts on these neurons to induce Fos expression and stimulate the release of oxytocin from the dendrites of these neurons, in a Ca^{2+} dependent manner, as intracellular Ca^{2+} mobilization by α-MSH primes the dendritic store of oxytocin for release and then triggers release.[308,310] It has been proposed that oxytocin released from the dendrites of magnocellular oxytocin neurons can diffuse in brain extracellular fluid to reach nearby neurons in the VMN, where OTR are abundantly expressed, and alter their electrical activity.[311–313] Actions of oxytocin in the VMN have been suggested to contribute to regulation of appetite;[314] however, this remains to be established.

Leptin and Oxytocin Neurons

Leptin, secreted by adipose tissue and reflecting fat store size, can enter the brain via the choroid plexus, and oxytocin neurons express leptin receptors.[315]

DIO in rats, induced by feeding a high fat diet, involves reduced numbers of arcuate neurones,[269] and leptin resistance (i.e., obesity despite high circulating leptin levels, and lack of effect of administered leptin on food intake).[316] In such rats, i.c.v. injection of oxytocin (1 μg) before lights out reduces overnight food intake and body weight similarly in normal and DIO rats, as does intraperitoneal injection of up to 1000 μg/kg oxytocin,[a] which stimulates neurons in the NTS and area postrema in both DIO and control rats; this treatment is similarly effective in reducing food intake in obese rats genetically deficient in leptin signaling.[316] These findings are interpreted as reflecting failure, in DIO rats, of leptin to signal via α-MSH from arcuate nucleus POMC/CART neurons to parvocellular oxytocin neurons that project to the NTS.[316] It seems possible that leptin resistance involves failure to activate central oxytocin mechanisms, and that in pregnancy, a similar situation could be important in permitting increased food intake.

Appetite Peptides and Oxytocin Neurons in Pregnancy

The effects on oxytocin neurons of peptides that are involved in appetite regulation are altered in pregnancy.

LEPTIN

Given acutely by intravenous injection, leptin stimulates the electrical activity of magnocellular oxytocin neurons; this action is lost in late pregnant rats that are fasted overnight but not in fasted virgin rats.[266] Hence, leptin may normally recruit magnocellular oxytocin neurons to augment its anorectic actions and to stimulate postprandial natriuresis.[318] The loss of the oxytocin neuron responses to leptin in the fasted state in late pregnancy may contribute to increased appetite and sodium retention (see the section Osmoregulation in Pregnancy).

NEUROPEPTIDE Y (NPY)

NPY, a 36–amino acid peptide produced by arcuate nucleus neurons, is a potent orexigen when given centrally.[319] It also stimulates oxytocin neurons, which express NPY receptors;[320] this action is lost in late pregnancy in rats, yet its orexigenic action is intact.[259] Hence, this loss of oxytocin neuron responses to NPY with maintained orexigenic action will favor increased food intake in pregnancy.

[a] for comparison, i.v. infusion of 1 μg over 2 h is sufficient to drive delivery of the litter in a late pregnant rat.[317] At the supraphysiological ECF concentrations of oxytocin expected after an intraperitoneal injection of 1000 μg/kg oxytocin (c. 5000 ng/ml: >5000-fold greater than maximal endogenous concentrations[297]) a small (but physiologically significant) percentage may cross the blood–brain barrier.[297] It needs to be tested whether effects of peripherally administered oxytocin in the brain are through central oxytocin receptors.

NESFATIN-1

Nesfatin-1 (also called nucleobindin 2, NUCB2-encoded satiety and fat-influencing protein) is a recently identified potent anorexigenic 82–amino acid peptide.[321–323] It is expressed in neurons in brain regions involved in regulation of appetite and metabolism,[323] in subcutaneous adipocytes, and in gut;[322] it is also present in the circulation and can cross the BBB.[324]

Released from arcuate nucleus POMC/CART neurons, probably by dendrites,[325] nesfatin-1 inhibits nearby NPY/AgRP neurons.[305,326] Nesfatin-1 is also co-expressed in oxytocin neurons and PVN CRH neurons, and expression in oxytocin neurons decreases with fasting and increases with refeeding.[327] Melanocortin signaling is involved in nesfatin's anorexic actions, and these are blocked by central administration of an oxytocin or a CRH-R2 antagonist, indicating mediation of nesfatin actions by α-MSH and oxytocin and CRH (or a urocortin).[305,328] Clearly, reduced production and action of nesfatin-1 on networks regulating appetite could support increased food intake in pregnancy, but this has not been reported.

RELAXIN-3

This peptide, a member of the relaxin family (see the section Osmoregulation in Pregnancy),[73,74] is expressed in the brain[329] and acts via the RXFP3 receptor, inhibiting adenylate cyclase—opposite to relaxin actions via RXFP1.[69,70,330] Relaxin-3 increases food intake and weight gain, acting via the PVN, possibly via reduced oxytocin production[331]; while human relaxin-2 (rat relaxin has similar reproductive biological activity)[332] has opposite effects.[333] Any role of relaxin-3 in increased appetite in pregnancy is presently unknown, however, the inhibitory actions of relaxin-2 on eating cannot explain increased food intake in pregnancy.

Oxytocin and Food Intake in Pregnancy: Oxytocin Resistance?

When food is returned after 24 h food deprivation, mid-pregnant rats start to eat more quickly, eat more, and spend more time eating than virgin rats.[109] Central infusion of an oxytocin antagonist before food is returned increases food intake over the next 24 h in virgin, but not in pregnant, rats.[109] Conversely, i.c.v. oxytocin infusion (1 μg; n.b. 500-fold greater than the dose that triggers coordinated burst firing of oxytocin neurons in suckled lactating rats[334]) reduces food intake in virgin rats after fasting for 24 h, but has no such effect in pregnant rats.[109] Together, these experiments indicate a lack of oxytocin anorectic activity in pregnancy. This may be the result of a lack of stimulation of oxytocin neurons during eating (e.g., by circulating leptin or CCK, or by NPY released in the brain, reduced OTR expression, or post-OTR signaling mechanisms in appetite-regulating regions).

OXYTOCIN RECEPTORS (OTR)

Expression of OTR (*Oxtr*) mRNA or oxytocin binding sites in the VMN are not altered in mid-pregnancy but are increased by the completion of parturition, probably consequent on estradiol/interleukin-6 (IL-6) action.[313] OTR are also expressed in the NTS, PVN, and SON, and *Oxtr* mRNA expression in the SON and NTS (but not in the PVN) is increased by the end of pregnancy.[303] OTR binding is increased in the PVN and SON after mid-gestation, compared with ovariectomized controls (in which estrogen and progesterone treatment mimic the changes observed in the SON but not the PVN in late pregnancy).[335] However, intact rather than ovariectomized controls are appropriate to seek changes due to pregnancy. Evidently, there is no reduced OTR expression in the VMN, NTS, PVN, or SON that might explain increased appetite in pregnancy.

NUCLEUS TRACTUS SOLITARII (NTS)

Some PVN oxytocin neurons project to the NTS, where oxytocin receptors are expressed,[298,303,336] and oxytocin release here interacts with CCK to enhance satiety (Figure 44.3). This release of oxytocin in the NTS following a meal is stimulated by noradrenergic neurons in the NTS that are excited by CCK and project to the oxytocin neurons in the PVN.[288,337,338] Interruption of this NTS-PVN circuit by ablating NTS neurons expressing OTR with a toxin (oxytocin-saporin conjugate) attenuates anorectic effects of CCK.[339] In late pregnancy, this NTS-PVN circuit may be suppressed by enhanced production of enkephalins by NTS neurons that presynaptically inhibit noradrenaline release in the PVN, so that CCK less effectively stimulates oxytocin neurons.[24,340]

Gastrointestinal Peptides

Cholecystokinin (CCK)

CCK secreted by the I cells in the small intestine acts centrally after a meal to limit meal size, synergizing with leptin to reduce food intake.[341] The central short-term anorectic (satiety) actions of CCK are mediated by vagal afferents and the area postrema and NTS in the medulla oblongata (Figure 44.3). CCK and leptin each electrically excites nodose ganglion neurons, and they synergize via separate receptors (CCKAR and LEPRb) on their peripheral nerve terminals and via converging postreceptor signaling mechanisms expressed by these neurons.[342]

In the NTS, CCK excites A2 noradrenergic neurons[343–347] and several types of neuron that produce peptides that modify eating, e.g., PRP, NPY, POMC, glucagon-like peptide-1 (GLP1, an anorexigen).[348] A2 neurons are important for the anorectic actions of CCK, and for activation of PVN oxytocin neurons by CCK.[337]

Prolactin-Releasing Peptide

Prolactin-releasing peptide (PrRP; 20 amino acids) is a member of the arginine and amidated phenylalanine (RFamide) peptide family.[349,350] It was identified originally as a ligand for an orphan G-protein coupled receptor (GPR 10, hGR3, or UHR-1).[349] PrRP was so named because it was found to stimulate the secretion of prolactin from lactotrophs in vitro,[351] but this is now not considered to be its function (see the section The Prolactin System: Preparation for Lactation). Instead, the function of PrRP in the brain is to negatively regulate appetite.[352–354] PrRP is produced by A1 and A2 noradrenergic neurons in the NTS, which project to the hypothalamus (including the PVN, SON, and dorsomedial region) and limbic regions, and it is also produced by neurons in the dorsomedial hypothalamus.[350,355]

Notably, PrRP-expressing neurons are activated by food intake or i.v. CCK,[345,353] and blocking central PrRP actions leads to hyperphagia.[345] There is evidence that the inhibitory actions of PrRP are mediated by CRH and oxytocin neurons.[356–358] When given by i.c.v. injection, PrRP stimulates secretion of prolactin, but it also stimulates the secretion of several other pituitary hormones including oxytocin.[359] Interestingly, NTS PrRP neurons express ER, and estradiol positively regulates NTS PrRP expression.[360,361] Hence NTS PrRP neurons might mediate the inhibitory actions of estrogen on appetite. PrRP mRNA expression in the NTS has been reported to be increased substantially in pregnancy in the rat[360] but not in the mouse,[362] and, in contrast, expression in the NTS is reduced in lactation.[353] As PrRP reduces appetite, and mRNA expression is increased in pregnancy, it seems that increased appetite in pregnancy is not explained by decreased PrRP production.

Projection targets of PrRP neurons include the PVN and SON, and they provide the route for circulating CCK to activate, for example, oxytocin neurons.[358] In nonpregnant rats, CCK given intravenously excites SON magnocellular oxytocin neurons, via a noradrenergic (A2) input from the NTS,[41,363] which stimulates somato-dendritic oxytocin release in the SON; this may be important in mediating anorectic actions of CCK.[364] In the PVN, systemic CCK also activates neurons expressing CART,[365] p-mammalian target of rapamycin (p-MTOR, associated with appetite suppression), a serine/threonine-kinase (expressed in oxytocin neurons)[366] and nesfatin (co-expressed with CRH or oxytocin).[367]

Activation by CCK of pPVN oxytocin neurons that project to the NTS is important in reinforcing the satiety actions of CCK, and these neurons are important in potentiating the anorectic actions of CCK by leptin.[288,338] CCK also alters the firing activity of VMN neurons, predominantly inhibiting, but whether this involves oxytocin is not known.[368]

Leptin-CCK Interactions

Synergy between CCK and leptin, as seen in vagal afferents, does not extend to actions in the PVN or SON, indicating that leptin acts also by another pathway[369] or perhaps directly on magnocellular oxytocin neurons.[266]

CCK is consistently found not to activate arcuate neurons, but leptin signaling in the arcuate nucleus is important for normal satiety actions of CCK via the NTS, perhaps via α-MSH/MCR4 signaling in the PVN or lateral hypothalamus.[370] Also important for satiety actions of CCK are interactions between α-MSH/MCR4 signaling and vagal input, via N-methyl-D-aspartate (NMDA) receptors, in the NTS.[344,371] CCK activates the ERK cascade in NTS neurons, which is necessary for the satiety effects of CCK[372] and for convergence with α-MSH/MCR4 signaling.[373] Notably, leptin evidently acts directly on NTS POMC neurons,[374] which, together with neurons expressing GLP1 or CCK, are the only NTS neurons that express LEPRb.[375] Leptin does not interact with CCK at the level of the VMN.[368]

CCK Resistance in Pregnancy

In the context of leptin resistance, CCK does not reduce food intake in pregnancy.[376] This may be because CCK is less effective in activating oxytocin neurons in late pregnancy,[377] which likely involves reduced noradrenaline release in the SON.[378] The reduced activation of NTS neurons by CCK in day 14 pregnant rats and the absence of activation of SON and PVN neurons (contrasting with CCK's actions in nonpregnant rats)[376] support this explanation. Importantly, CCK activates only oxytocin neurons in the SON,[41] and in the PVN it activates parvocellular and magnocellular oxytocin neurons, but not in day 14 pregnant rats.[376] Central (possibly in the NTS) or vagal leptin resistance (as seen in DIO),[187,188] may underlie the lost action of CCK on oxytocin neurons in late pregnancy. It is also possible that this is explained by μ-opioid inhibition acting on oxytocin neurons, or on the noradrenergic input from NTS A2 neurons, which emerges in late pregnancy and inhibits dendritic oxytocin release as well as firing rate responses.[24,340] This inhibited stimulation of oxytocin neurons by CCK in late pregnancy may have the same consequences for increased appetite (and sodium retention) as the reduced leptin sensitivity of oxytocin neurons discussed previously.[266]

Like CCK, peptide YY (PYY) is an anorexigen secreted by the intestine after a meal, and PYY levels are increased in pregnant rats.[217] The finding in pregnancy of increased circulating levels of PYY (an anorexigen), together with increased food intake, indicate evident resistance to the anorexic actions of PYY in pregnancy; whether this involves altered responsiveness of NPY neurons to inhibitory actions of PYY is not known.

Reward Circuitry: Opioids and Food Intake

Hedonic appetite is motivation that may be a coping response to stress to eat preferentially palatable, energy-rich food for pleasure, in excess of need to maintain metabolic homeostasis.[379,380] Hedonic appetite is regulated by the mesolimbic dopaminergic reward system, comprising the ventral tegmental area (VTA) and nucleus accumbens (NAc) with connections to the central nucleus of the amygdala (CeA), frontal cortex, and bed nucleus of the stria terminalis (BNST) (see the section Maternal Behavior).[381] This network nonetheless has important interactions with the homeostatic networks, based in the NTS and hypothalamus. Hence, for example, ghrelin has appetite-stimulating actions through both the reward and hypothalamic networks.[146] The reward network is also key in organizing maternal behavior (see the section Maternal Behavior).[382]

It has been known for over 40 years that endogenous opioids are involved in short-term positive regulation of appetite, following reports of inhibitory actions of naloxone, an opioid receptor antagonist, and subsequent reports of stimulatory actions of centrally administered opioid peptides.[383,384] Although all three opioid receptor types are involved, predominant actions via μ-opioid receptors at multiple sites in pathways regulating hedonic appetite have been identified.[381] Within the hedonic reward system, a μ-selective synthetic enkephalin-like opioid [D-Ala(2), NMePhe(4), Gly-ol(5)]enkephalin (DAMGO) acts in both the VTA and NAc to increase feeding, and naltrexone (long-acting form of naloxone) given into either site blocks the effects of DAMGO in the other.[385] Similar experiments demonstrate μ-opioid-mediated interactions between the VTA and PVN to increase feeding.[386] Moreover, DAMGO given into the CeA inhibits CeA neurons, then excites NAc (shell) neurons and increases feeding.[387] In particular, DAMGO injection into the NAc stimulates high fat diet intake, but this effect involves excitatory projections to hypothalamic orexin neurons and requires orexin action in the NAc or VTA;[388] these findings illustrate the interaction between hypothalamic homeostatic and mesolimbic hedonic regulation of food intake.[381]

Opioids and the NTS

Importantly, the reward network connects to the hypothalamic network via the lateral hypothalamus (LHA) and the NTS (Figure 44.3). Microinjection experiments have shown reciprocal interactions between μ-opioid actions on feeding in the NTS and VTA.[389] In the NTS, POMC neurons (identified by green fluorescent protein (GFP) expression in transgenic mice) are activated by CCK (local or systemic) and inhibited by met-enkephalin. These POMC neurons may have local effects, via α-MSH and MCR4 in the NTS, and their inhibition by enkephalin could contribute to stimulation of feeding through NTS connections to the CeA and NAc.[343] As discussed elsewhere (see the sections The Hypothalamo-Pituitary-Adrenal Axis and Magnocellular Oxytocin Neuron System in Pregnancy), pro-enkephalin-A (*Penk-a*) gene expression is increased in the NTS in late pregnancy,[21] but any impact on hedonic food intake has not been studied.

Opioids and Hypothalamic Circuitry

Stimulation of feeding by NPY injection into the PVN is inhibited by naltrexone injection into the medial NTS, reflecting opioid support of NPY actions via reciprocal PVN-NTS interactions.[390] Moreover, microinjection studies of effects of naltrexone and DAMGO on feeding show reciprocal μ-opioid-mediated stimulation in the PVN and VTA.[386] The powerful stimulatory action on feeding of centrally administered NPY is reduced by μ, δ, and κ-opioid receptor antagonists.[391] Similarly, stimulation of feeding by i.c.v. administration of orexin, but not MCH, is reduced by naloxone.[392] In late pregnancy, i.c.v. injection of NPY still evokes an eating response similar to that in virgin rats,[259] and the inhibition by naloxone of NPY-stimulated feeding seen in nonpregnant rats[391] is exaggerated in late pregnancy (Bales J, Brunton PJ, Russell JA, unpubl.). This may reflect upregulation of endogenous opioid mechanisms in the brain, including in the NTS, in late pregnancy.[21,24,340,393,394]

ARCUATE NUCLEUS

The number of arcuate neurons expressing POMC mRNA (in the caudal arcuate) or immunoreactive for β-endorphin is increased near the end of pregnancy, and the number of β-endorphin fibers in the SON is also increased. Estradiol and progesterone actions may underlie this change as arcuate β-endorphin neurons express PR.[244,395–397] Whether this reflects a switch to processing POMC to β-endorphin, with reduced α-MSH production is not known. Such a switch could enhance opioid inhibition of oxytocin neurons, including dendritic release, as emerges near the end of pregnancy,[24] and might reduce stimulation of dendritic oxytocin release by α-MSH.[308] However, β-endorphin knockout mice are hyperphagic, and retain normal feeding responses, respectively, hyper- and hypophagic, to other opioids and to the antagonist naloxone.[398] Together, these findings do not indicate a role for β-endorphin regulation of oxytocin neurons in appetite control, at least without pregnancy.

ENKEPHALINS

Another way in which opioids may increase appetite is by inhibiting oxytocin release in response to anorexigens. During the last half of pregnancy, in rats, magnocellular oxytocin neuron responses to input from the NTS (including stimulation of this input by CCK) are inhibited by

emergence of opioid inhibition.[24,394,399] Importantly, central release of oxytocin is restrained by an endogenous μ-opioid mechanism in late pregnancy.[24] This is likely to be a result of stimulation by allopregnanolone of increased expression of *Penk-a* and μ-opioid receptor (MOR, *Oprm1*) mRNAs in NTS neurons, with consequent presynaptic inhibition of noradrenaline release in the PVN and SON.[21,340]

In addition, allopregnanolone, present in the brain at high levels in late pregnancy, directly inhibits activity of magnocellular neurons.[400] Clearly, this could contribute to reducing oxytocin neuron responses to anorexigens; however, testing an allopregnanolone-opioid link by systemic naloxone administration results in reduced food intake, similar to that seen in nonpregnant rats.[109] This outcome is not conclusive, as systemic naloxone reduces hedonic appetite, as discussed earlier, which may obscure a selective action on food intake of blocking the established inhibitory action of opioid on dendritic oxytocin release in pregnancy.[24]

Summary and Conclusions

Possible changes in pregnancy to explain increased food intake and consequent increased energy storage have been considered in the context of the neural circuitry in the hypothalamus and brain stem that homeostatically regulates appetite, the mesolimbic circuitry regulating hedonic appetite, and the actions of circulating hormones on or in this circuitry. In early pregnancy, increasing progesterone secretion stimulates food intake (offsetting an inhibitory action via stimulation of arcuate POMC neurons by estrogen). The hypothalamic circuitry involves neurons that use many signaling peptides and GABA to increase or decrease food intake and energy storage and utilization. In mid- to late pregnancy, first-order arcuate NPY/AgRP neurons (which increase food intake and energy storage) may be more active. These neurons are normally negatively regulated by leptin, secreted in increasing amounts as the adipose store increases. At the level of the hypothalamus there is leptin resistance (reduced postreceptor signaling) in pregnancy, but contrary to expectation, signaling (STAT3 induction) by leptin is evidently intact in the arcuate NPY/AgRP and POMC (α-MSH) neurons (which decrease appetite and increase energy expenditure). Other post-LEPRb signaling pathways still need to be examined. Instead, leptin resistance (with reduced LEPRb expression) is found in second-order neurons (i.e., targets for α-MSH), in the PVN and VMN (satiety center) during pregnancy, which seems to be induced by the high levels of prolactin. Importantly, the anorectic actions of α-MSH are lost (α-MSH resistance) in pregnancy. Oxytocin has central anorectic actions; pPVN neurons project within the brain, including to the NTS, while magnocellular oxytocin neuron dendrites release oxytocin (when stimulated by α-MSH), which may diffuse to target neurons expressing OTR. In pregnancy, suppression of responses of pPVN and magnocellular PVN and SON neurons to feeding stimuli (e.g., NPY, CCK) and leptin is a strong candidate mechanism for increased food intake in pregnancy, as is resistance to the anorectic actions of oxytocin. The NTS integrates vagal input (e.g., conveying signals about circulating CCK) and input from the PVN (e.g., from oxytocin neurons), and NTS neurons signal satiety via noradrenaline, GLP1, and PrRP to the hypothalamus (e.g., to oxytocin neurons). Oxytocin neurons are evidently resistant to excitation by CCK signaling via the NTS in late pregnancy, which will act to increase eating. This resistance may involve reduced CCK action on vagal afferents as a consequence of leptin resistance and loss of synergy with leptin. Also, CCK resistance may be a result of activation of an endogenous opioid inhibitory mechanism on NTS inputs to oxytocin neurons.

Future Perspectives

Many aspects of altered appetite regulation in pregnancy remain to be investigated. These include the possibility of: altered serotonin and eCB actions in the hypothalamic homeostatic circuitry regulating appetite and metabolism; a role for low adiponectin levels in pregnancy in central control of altered energy balance; a role for the high levels of allopregnanolone; reduced nesfatin and increased relaxin-3 production in the brain; and reduced GLP1 production in the NTS and plasticity of PVN-NTS connections in pregnancy, especially of oxytocin actions. The significance of reported increased expression of PrRP (an anorectic peptide) in the NTS in pregnancy needs clarification. Testing roles for upregulated endogenous opioid expression in resetting the homeostatic and hedonic neural networks regulating appetite and metabolism is difficult because of opposite, but complementary, opioid actions on these networks. This will need focal exploration of possible roles of opioids at specific nodes in these networks with available novel techniques. It is possible that the experience of increased food intake and metabolic changes in pregnancy leaves epigenetic traces in hypothalamic and mesolimbic circuitry as in DIO, with enduring effects on appetite and metabolism.[401]

THE PROLACTIN SYSTEM: PREPARATION FOR LACTATION

In the 1920s, several researchers studying several species established that anterior pituitary extracts could induce lactogenesis.[402] Subsequently, Riddle et al.[403] isolated prolactin from the anterior pituitary and reported that it acted distinctly differently from other hormones known at the time. Prolactin is a peptide hormone (197 amino acids in rats; 199 amino acids in humans)[17,403] produced by lactotroph cells in the anterior pituitary gland.

Prolactin has an essential role in lactation by stimulating milk production and secretion in the mammary gland alveoli; in pregnancy prolactin acts on the alveoli to prepare for milk production. Accordingly, the secretion of prolactin is greatly increased in late pregnancy in the rat (Figure 44.1), and in women prolactin secretion increases continually through pregnancy (in late pregnancy by c. 20-fold from prepregnancy values).[404]

In the rat prolactin has an essential role in early pregnancy in preventing luteolysis, and hence in sustaining progesterone secretion for the pregnancy. Prolactin has many other actions,[17,405] of which a role in the initiation of maternal behavior and in appetite regulation are relevant to this chapter (see the sections Food Intake and Metabolism in Pregnancy and Maternal Behavior).

Prolactin secretion is under the control of the brain through the production and release of prolactin release inhibiting (PIF) and prolactin releasing factors (PRF; Figure 44.5). Unlike other anterior pituitary hormones, the predominant control of prolactin secretion is by a PIF. This is well established to be dopamine, produced and secreted by hypothalamic dorsomedial arcuate nucleus neurons (A12, tubero-infundibular dopamine: TIDA neurons; tubero-infundibular refers to this brain region in humans). Without pregnancy and lactation, the secretion of prolactin is restrained by tonic dopamine secretion into the hypothalamo-hypophysial portal system.[16] Dopamine is synthesized from tyrosine, which tyrosine hydroxylase (TH; rate-limiting enzyme, constitutively active in TIDA neurons) converts to L-dihydroxyphenylalanine (L-DOPA), from which dopamine is formed by DOPA decarboxylase action. DOPA is stored in secretory granules and released by exocytosis from axon terminals in the median eminence when the neurons are active. Dopamine is inactivated enzymatically by monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT), and there is a reuptake mechanism.[16] In the anterior pituitary dopamine acts primarily on D2 receptors (a 7-transmembrane domain G-protein linked receptor) on lactotrophs, which mediate continual inhibition of prolactin production.[16] Hence, prolactin secretion is stimulated when dopamine secretion by TIDA neurons is inhibited, or when secretion of a PRF is increased, or both occur (see also Chapter 12).

There are major adaptive changes in the brain in pregnancy in the mechanisms regulating prolactin secretion that permit exposure of peripheral and brain targets to high levels of prolactin in early and late pregnancy, while there are high levels of placental lactogen from mid-pregnancy (Figure 44.1).[406]

Regulation of TIDA Neurons

Multiple neurotransmitters and modulators are involved in regulating TIDA neurons (Figure 44.5),[17] as is

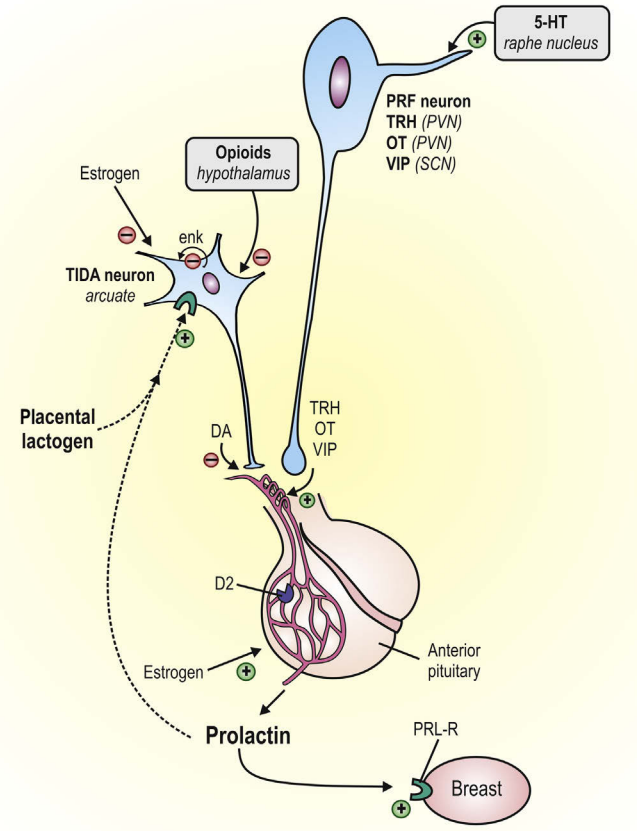

FIGURE 44.5 The hypothalamo-pituitary prolactin system. In nonpregnant, nonlactating animals prolactin secretion is under tonic inhibition by dopamine (DA) produced by tubero-infundibular dopamine (TIDA) neurons in the hypothalamus and secreted into the hypothalamo-hypophysial portal system to act on dopamine D2 receptors on the lactotrophs. TIDA neurons are inhibited by endogenous opioid peptides, which can thus stimulate prolactin secretion. There are several candidate prolactin releasing factors (PRFs), including thyrotropin-releasing hormone (TRH) and oxytocin (OT) produced in the parvocellular division of the paraventricular nucleus (PVN) and vasoactive intestinal peptide (VIP) produced by neurons in the suprachiasmatic nucleus (SCN), which stimulate prolactin secretion. PRF neurons are activated by serotoninergic (5-HT) inputs from the raphe nucleus. Estrogen sensitizes the pituitary to release prolactin and also inhibits dopamine synthesis in TIDA neurons. Prolactin can enter the brain by being transported across the blood–brain barrier via a specific transport mechanism. Prolactin feedback is important in maintaining the rhythm in prolactin secretion once it is established in early pregnancy (see Figure 44.1). Prolactin acts via prolactin receptors (PRL-R) in a short feedback loop to stimulate dopamine production by TIDA neurons. From mid-pregnancy increasing production of placental lactogen (not controlled by the brain) acts like prolactin to stimulate TIDA neurons, which eventually become resistant to stimulation, so that prolactin secretion surges near the end of pregnancy (Figure 44.1). Enkephalins (enk) produced by the TIDA neurons near the end of pregnancy may contribute to this surge by auto-inhibiting the TIDA neurons. In late pregnancy prolactin prepares the mammary glands for milk secretion.

the case for other hypothalamic neuroendocrine neurons. Hence, many of these agents given into the brain may alter prolactin secretion through such actions on TIDA neurons.

Prolactin Receptor

There is a single prolactin receptor (PRL-R, found as the long or short form), which is a membrane-bound protein of the cytokine receptor superfamily.[407] The long form (PRL-R_L, the active signaling form) and the short form of the PRL-R (PRL-R_S), result from alternative mRNA splicing from a single gene; the two forms of the receptor have different intracellular domains.[407] Prolactin has important "short-loop" feedback actions on TIDA neurons, via PRL-R_L, increasing *Th* mRNA expression and resulting in increased dopamine production and hence decreased prolactin secretion.[408] Phosphorylated signal transducer and activator of transcription 5b (pSTAT5b) is an essential mediator of prolactin actions on TIDA neurons,[409,410] and, assessed by pSTAT5 induction, these neurons show greater sensitivity to prolactin than all other neurons that respond to prolactin.[265]

Placental Lactogen

In late pregnancy, placental lactogen action results in a sustained increase in dopamine secretion, hence inhibiting prolactin secretion.[406]

Endogenous Opioid Peptides

TIDA neurons are inhibited by endogenous opioid peptides, which thus stimulate prolactin secretion; both μ- and κ-opioid receptors are involved in pregnancy.[411,412] Indeed, in early pregnancy nocturnal prolactin surges are blocked by naloxone, indicating an important role for an endogenous opioid mechanism in permitting the surges.[413] The identity of the opioid peptide mediating this action is not clear: TIDA neurons are contacted by dynorphin and met-enkephalin axons, especially the former.[414]

Estrogen

Exposure to estrogen induces a prolactin surge in the rat, which involves inhibition of TH in TIDA neurons.[415]

Prolactin Releasing Factors

By definition, a hypothalamic releasing factor is secreted from neuroendocrine neuron axon terminals adjacent to primary capillaries in the median eminence of the hypothalamo-hypophysial portal vessel system and acts in the anterior pituitary gland to regulate secretion of an anterior pituitary hormone.[416,417] Hence a range of criteria, including functional importance, must be satisfied to identify a hypothalamic product as a releasing factor or a release-inhibiting factor. There are several candidate PRFs: thyrotropin releasing hormone (TRH), oxytocin, vasoactive intestinal peptide (VIP), and prolactin releasing peptide (PrRP).[17]

Thyrotropin Releasing Hormone

Besides being a hypothalamic-releasing factor that stimulates thyrotropin (thyroid stimulating hormone; TSH) secretion from the anterior pituitary (and the first releasing factor to be characterized),[416] TRH also stimulates prolactin secretion and the secretion of several other hormones.[418] In mammals TRH is produced by neurons in several hypothalamic areas, but TRH neurons in the pPVN are those involved in control of PRL secretion.[419,420] As a PRF, TRH is distinctive as it can stimulate prolactin secretion in the presence of dopamine inhibition.[421]

Oxytocin and Vasoactive Intestinal Peptide

These peptides are both present in portal blood and can stimulate prolactin secretion from lactotrophs in the absence of dopamine inhibition.[421] Vasoactive intestinal peptide (VIP) is also produced by suprachiasmatic nucleus (SCN) neurons that project to TIDA and oxytocin neurons, and these central projections may mediate the important actions of VIP in the regulation of prolactin secretion, acting via the oxytocin neurons.[422]

Prolactin Releasing Peptide

PrRP was so named because it was found to stimulate the secretion of prolactin from lactotrophs in vitro,[351] but this is a misnomer as it is not present in the ventral hypothalamus, where classical releasing factors are found, and it does not satisfy criteria for a releasing factor.[350] However, PrRP has important functions in the brain; it is produced by NTS neurons[355] and is involved in HPA axis stress responses and in appetite control (see the section Food Intake and Metabolism in Pregnancy).[354,423]

Control of Prolactin Surges in Early Pregnancy

As a result of copulation in the rat, the lifespan of the corpora lutea is extended by the reflex stimulation of secretion of a luteotropic complex (luteinizing hormone and prolactin) by the anterior pituitary gland. A circadian pattern of once-daily (diurnal) and once-nightly (nocturnal) pulses of prolactin is triggered, which contributes to maintenance of the corpora lutea for the first half of pregnancy (10–12 days; Figure 44.1). If the mating is sterile, the same events occur, leading to pseudopregnancy.[424] This stimulation of prolactin secretion in early pregnancy is considered to require action of PRF(s) as well as withdrawal of inhibition by dopamine.[17]

Studies to understand the mechanisms controlling prolactin secretion in early pregnancy in the rat have involved experimental models with normal or sterile mating, cervical stimulation (simulating mating), and hormone manipulations in ovariectomized rats. Measurements in hypothalamo-hypophysial portal blood in ovariectomized rats made hyperprolactinemic by implanting donor anterior pituitaries under the renal capsule indicated

stimulation of dopamine and oxytocin release, and inhibition of VIP release.[425] Outcomes of studies on the aforementioned models indicate that several mechanisms are involved in the control of prolactin release in early pregnancy. Experimental data from studies on VIP, oxytocin, and TIDA dopamine neuron activities have been mathematically modeled to understand how interactions among these neurons act as a pacemaker and organize the circadian pattern of prolactin secretion that is seen during early pregnancy or in pseudopregnancy.[426–428]

Oxytocin

A key response to copulation is secretion of oxytocin from the posterior pituitary gland, and simulation of this oxytocin release (5 μg i.v., a large dose, see footnote a) triggers the daily and nightly surges of prolactin seen in early pregnancy.[427,429] However, it seems unlikely that oxytocin action on the lactotrophs is responsible for triggering formation of the central "memory" that sustains this pattern; nor is central action of prolactin likely, nor an action of TRH on lactotrophs.[430,431] Evidently, the action of oxytocin in triggering this neuroendocrine memory depends on intact pelvic nerves, as does the triggering of the luteotropic prolactin pulses.[431] Whether this means that uterine responses to oxytocin are necessary for triggering the diurnal prolactin pulses by copulation is not clear. The central pathway for effects of cervical stimulation involves brain stem noradrenergic input to the PVN.[432] The central mechanism for the "memory" that patterns prolactin secretion in early pregnancy in the rat is not yet clear,[428] although central release and action of oxytocin in the VMN is evidently important.[433]

Vasoactive Intestinal Peptide

The prolactin secretory pattern induced by pharmacologically reducing dopamine tone that is similar to the pattern in early pregnancy is determined by changes in circadian VIP release by SCN neurons in the hypothalamus (i.e., VIP not acting as a releasing factor), which project to and inhibit TIDA dopamine neurons.[434,435] Importantly, the diurnal and nocturnal prolactin increases involve VIP stimulation of oxytocin secretion, and the diurnal prolactin surge involves stimulation of oxytocin secretion also by serotonin (acting in the hypothalamus).[426,436]

Thyrotropin Releasing Hormone

Exogenous TRH has been reported to most effectively stimulate prolactin during the peaks in early pregnancy, and is less effective from mid-pregnancy.[437] However, the circadian prolactin pattern in early pregnancy is not induced by TRH.[431]

Negative Feedback by Prolactin

Prolactin can enter the brain by being transported across the blood–brain barrier into CSF via a specific transport mechanism, involving PRL-R expressed in the choroid plexus.[438,439] PRL-R (long isoform) expression in the choroid plexus is increased by high levels of estrogen in pregnancy, which facilitates the entry of prolactin into the brain.[440,441]

TIDA Neurons

Acting via TIDA neurons, such that prolactin stimulates dopamine production, prolactin feedback is well established to be important in maintaining the rhythm in prolactin secretion once it is established in early pregnancy.[427,429,430] Prolactin acts by upregulating TH gene expression, and by inducing phosphorylation of TH, prolactin prevents inhibition of TH by dopamine.[410] Although the "memory" triggered by oxytocin administration in ovariectomized rats does not involve central prolactin action,[431] in mated rats central prolactin action does seem to be responsible for triggering the surges of prolactin.[430]

Oxytocin Neurons

The models of regulation of oxytocin neurons in the context of control of prolactin secretion involve prolactin stimulating[429] or inhibiting oxytocin neurons.[428] Neurons in the PVN and SON, and specifically most oxytocin neurons, express PRL-R,[427,439,442] with increased numbers of oxytocin neurons in the PVN expressing PRL-R in pregnancy.[267] Prolactin has direct prolonged inhibitory actions on the electrical activity of PVN and SON magnocellular oxytocin neurons, in vivo and in vitro;[267,443] hence prolactin will act to inhibit oxytocin secretion from the posterior pituitary as this is tightly related to action potentials arriving from the oxytocin neuron cell bodies. Nonetheless, in vitro prolactin has been found to increase oxytocin mRNA content in the basal hypothalamus.[444] There may be other prolactin actions on oxytocin neurons via postreceptor signaling mechanisms; the long form of PRL-R signals via the JAK/STAT pathway, and magnocellular oxytocin neurons, express STAT5.[445] However, at least without pregnancy, oxytocin neurons demonstrate less sensitivity to prolactin than TIDA neurons, as assessed by STAT5 induction; interestingly, pPVN oxytocin neurons are more sensitive to prolactin than magnocellular oxytocin neurons.[265]

Control of Prolactin Secretion from Mid-Pregnancy: Preparation for Lactation in Late Pregnancy

Placental Lactogens Take Control

In mid-pregnancy the placenta begins to produce lactogens (I and II; Figure 44.1, Figure 44.5), which are chemically similar to prolactin, with similar actions via prolactin receptors, and henceforth placental lactogens predominate.[17] As placental lactogen production is not regulated by

the maternal brain, its actions on the maternal mechanisms regulating prolactin secretion become important. Hence central actions of increasing levels of lactogens on TIDA neurons, stimulating dopamine release, lead to suppression of prolactin secretion from around mid-pregnancy (Figure 44.1).[446] Such stimulation of TIDA neurons by placental lactogen[447] is incorporated in a mathematical model of changes in prolactin control in pregnancy.[427]

Prolactin Secretion Returns in Late Pregnancy

During the night before parturition, prolactin secretion surges again (Figure 44.1). This late pregnancy pattern in the rat is more like that seen throughout pregnancy in women.[448]

ESCAPE FROM INHIBITION

This prolactin surge in the rat indicates escape from the sustained inhibition by dopamine, due to reduced responsiveness of TIDA neurons to stimulation by placental lactogen, and consequent lack of increased dopamine production with the increased prolactin secretion.[449–451] This reduced sensitivity to feedback actions of prolactin, involving loss of STAT5b induction by prolactin, and hence reduced dopamine production, is attributable to upregulation of suppressors of cytokine signaling (SOCS) proteins in the TIDA neurons.[452] Estrogen and progesterone are importantly involved in these changes: TIDA neurons express ERα and PR, without significant changes near the end of pregnancy.[453] Progesterone withdrawal near the end of pregnancy permits the high levels of estrogen and prolactin at this time to upregulate expression of three SOCS mRNAs (*Socs1*, *Socs3*, and cytokine-inducible SH2-containing protein, *Cish*) in the arcuate nucleus.[454] As STAT5b signaling is suppressed, induction by prolactin of SOCS mRNAs at this stage may involve signaling by alternative post-PRL-R mechanisms, for example, by MAPK.[454]

ENDOGENOUS OPIOID ACTIONS

Hyperprolactinemia, with progesterone support as in pregnancy, results in enkephalin gene expression in TIDA neurons, which may be involved in autoregulation of these neurons.[455,456] There are also dynorphin and β-endorphin fibers in the vicinity of TIDA neurons that may mediate opioid inhibition.[414] Strikingly, naloxone infusion in late pregnancy enhances TIDA neuron activity and blocks the prepartum surge in prolactin secretion.[457] Hence, opioid action on TIDA neurons has an important role in permitting the prolactin surge at the end of pregnancy, as well as permitting nocturnal surges at the beginning.[413]

POSSIBLE PRF ROLE

Removal of the posterior pituitary (including the intermediate lobe) prevents the prolactin surge at the end of pregnancy; this indicates that a PRF from the posterior pituitary might be important in driving the surge. This is not oxytocin but might be VIP.[458,459]

The mechanisms governing the final surge of prolactin secretion at the end of pregnancy appropriately ensure that sufficient prolactin is circulating to trigger bulk milk secretion and, after entry into the brain, with other mechanisms, promote maternal behavior after birth of the young.

Summary and Conclusions

The TIDA neurons form the hub in the hypothalamus through which prolactin secretion is predominantly regulated, with dopamine acting as a PIF. Without pregnancy, TIDA neurons are tonically active and prolactin stimulates these neurons, hence inhibiting its own release via this negative feedback loop. However, in early pregnancy in rats, TIDA neurons are inhibited by endogenous opioids, and PRF neurons are recruited to the hub. These oxytocin and VIP neurons sustain pulsatile prolactin secretion, which has important luteotropic actions (Figure 44.1). This pattern continues for a few days, reflecting a neuroendocrine memory for the initial trigger, copulation. Later in pregnancy, increasing placental lactogen secretion, which is not regulated by the brain, intercedes to stimulate TIDA neurons and thus suppress prolactin secretion. Subsequently, the TIDA neurons develop lactogen and prolactin resistance, which involves estrogen and progesterone actions, and in late pregnancy TIDA neurons express opioids, which are presumptively auto-inhibitory. Hence prolactin secretion finally surges at term.

Future Perspectives

PRP can stimulate prolactin release, but the location of PRP neurons in the brain does not fit a physiological role for PRP in direct regulation of prolactin secretion. It is an open question whether in pregnancy a specific prolactin-releasing factor is involved other than oxytocin and VIP (TRH is evidently not important). The importance and direction of prolactin actions on oxytocin neurons in pregnancy needs clarification, as does whether enkephalins expressed by TIDA neurons in late pregnancy auto-inhibit these neurons. The mechanisms in the hypothalamus involved in the neuroendocrine memory of mating await exploration. Computer-based modeling should continue to aid understanding of the changing control of prolactin secretion in pregnancy.

THE HYPOTHALAMO–PITUITARY–ADRENAL AXIS

The hypothalamo–pituitary–adrenal (HPA) axis plays a crucial role in restoring homeostasis following stressful stimuli. The concept of biological stress was first introduced

by Hans Selye in 1936,[460] who described the general adaptation syndrome, defined as "the sum of all non-specific, systemic reactions of the body which ensue upon long continued exposure to stress." In the early 1950s, Geoffrey Harris demonstrated in rabbits that stress-induced adrenocorticotropic hormone secretion was controlled by the hypothalamus acting through the hypophysial portal vessels of the pituitary stalk,[461] and soon after Guillemin (a former student of Selye) demonstrated the existence of a hypothalamic factor, coined corticotropin-releasing factor (CRF), that elicited ACTH release from the anterior pituitary gland in rats.[462] However, it was not until the early 1980s that the 41-amino acid peptide, CRF (or corticotropin releasing hormone; CRH) was finally identified and characterized by Wylie Vale and colleagues.[463] We now know that in response to stressors, whether physical or emotional, neurons in the medial parvocellular division of the PVN that synthesize CRH (and in some cases co-express arginine vasopressin; AVP) are activated and secrete CRH and/or AVP from their nerve terminals in the median eminence into the hypothalamo-hypophysial portal blood (Figure 44.6). CRH and AVP act synergistically on their receptors (CRH-R1 and V1b, respectively) in the anterior pituitary to stimulate ACTH secretion, which in turn triggers glucocorticoid (corticosterone or cortisol, depending on species) synthesis and secretion from the adrenal cortex. Glucocorticoids facilitate appropriate stress responses by promoting energy mobilization,[464] cardiovascular,[465] immune,[466] and behavioral responses,[467] as well as exerting negative feedback via glucocorticoid (GR; *Nr3c1*) and mineralocorticoid receptors (MR; *Nr3c2*) in the brain, or anterior pituitary (GR), to terminate the HPA axis response.[468]

Exposure to stress during pregnancy can affect the development of physiological systems in the offspring, resulting in increased susceptibility to cardiovascular[469] and metabolic disease[470] and to affective disorders in adulthood,[471] a phenomenon termed *fetal programming*. There are, however, "in-built" mechanisms that protect the fetus from this potentially detrimental programming. As a last line of defense, the placenta expresses 11β-hydroxysteroid dehydrogenase type 2,[472] an enzyme that by converting corticosterone to inert 11-dehydrocorticosterone, acts as a barrier to limit exposure of the fetus to circulating maternal glucocorticoids. A first line of defense is provided by reduced responsiveness of the maternal HPA axis. This acts to buffer the impact of stress, hence reducing fetal exposure to excessive glucocorticoids and minimizing the risk of detrimental programming,[471] while also promoting the anabolic adaptations in the mother necessary for successful pregnancy.[129]

Basal Activity of the HPA Axis in Pregnancy

During early pregnancy in rats, the circadian variation in ACTH and corticosterone secretion is suppressed

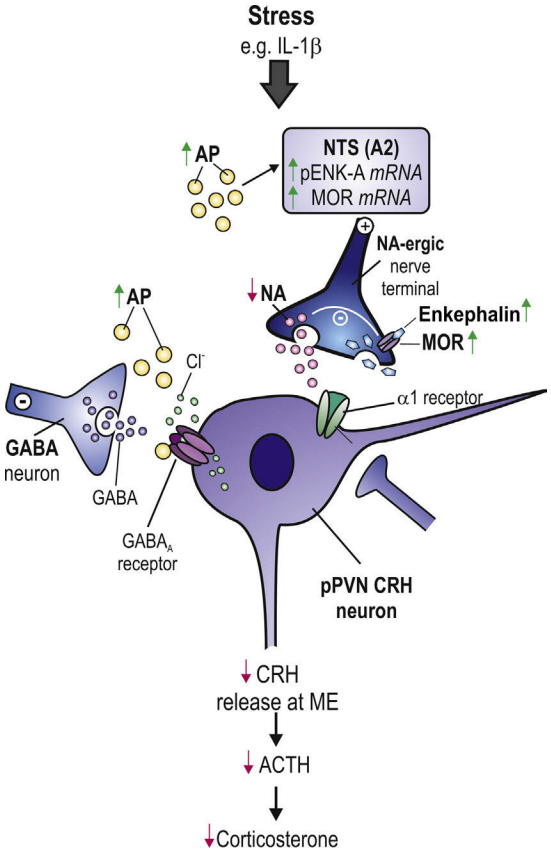

FIGURE 44.6 Allopregnanolone-opioid mechanisms involved in suppressed hypothalamo-pituitary-adrenal (HPA) axis responses to stress in pregnancy. Brain and circulating levels of allopregnanolone (AP) are increased (↑) in pregnancy (see Figure 44.1). In brain stem nucleus tractus solitarii (NTS) neurons, mRNA expression for proenkephalin-A (*Penka*) and μ-opioid receptor (MOR; *Oprm1*) is increased in pregnancy, as a result of increased AP production. Noradrenergic A2 neurons in the NTS project to parvocellular corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN). Noradrenaline (NA) released in the PVN excites the CRH neurons via α1 adrenoceptors, CRH is released at the median eminence (ME) and is carried in the hypothalamo-hypophysial portal system to stimulate anterior pituitary corticotrophs to secrete adrenocorticotropic hormone (ACTH), which stimulates glucocorticoid secretion (corticosterone in rodents; cortisol in humans) by the adrenal glands. In pregnancy, systemic interleukin-1β treatment (a stressor) fails to evoke noradrenaline release from the terminals in the PVN, hence ACTH and corticosterone secretion are not stimulated, unlike before pregnancy. This is a result of increased opioid inhibition (by enkephalin) acting presynaptically on the upregulated MOR, presumptively on the noradrenergic nerve terminals. In addition, AP may inhibit CRH neurons by positively modulating GABA inputs to the PVN via actions on $GABA_A$ receptors; AP prolongs the opening time of chloride (Cl^-) ion channels, enhancing inhibitory GABA neurotransmission.

and levels at the daily midpoint of the circadian range are reduced.[473] Salivary cortisol levels are also generally lower in women during early pregnancy compared with late pregnancy.[474,475] Reduced glucocorticoid secretion in early gestation has been suggested to facilitate implantation since elevated salivary cortisol levels in women at 1–3 weeks post conception have been associated with miscarriage.[476]

In the second half of pregnancy in the rat, basal levels of circulating corticosterone steadily increase, despite ACTH secretion remaining strongly suppressed.[473] This is a result of increased sensitivity of the adrenal gland to ACTH, probably as a consequence of estrogen actions on the adrenal cortex.[477,478] Similarly, basal cortisol levels progressively increase in the second half of gestation in women.[479]

Metabolic clearance of corticosterone is sustained at prepregnancy levels throughout gestation,[480] however, the total amount of free corticosterone declines in pregnancy, as a result of an increase in circulating corticosterone binding globulin (CBG), an adaptation seen in rats[481] and mice.[482]

In the brain, *Crh* and *Avp* mRNA expression in the PVN and *Gr* (*Nr3c1*) and *Mr* (*Nr3c2*) mRNA expression in the PVN and hippocampus are unaltered in early pregnancy.[483] Anterior pituitary ACTH content and the expression of mRNA for its precursor, POMC, is also unaltered in the first half of pregnancy.[484] However, in late pregnancy, basal expression of *Crh* mRNA and *Avp* mRNA in the pPVN are reduced,[483] concomitant with reduced median eminence CRH content and anterior pituitary *Pomc* mRNA and CRH receptor-type 1 (CRH-R1; *Crhr1*) mRNA expression.[484] These adaptations reflect reduced forward drive of the HPA axis in late pregnancy.

HPA Axis Responses to Stress in Pregnancy

In the first half of pregnancy in rodents, ACTH and corticosterone responses to acute stressors remain similar to those of nonpregnant females,[20,485,486] even though CRH release may already be reduced.[20] In contrast, the experience of "chronic stressful life events" during early pregnancy in women blunts peak salivary cortisol levels in morning samples, without affecting evening (nadir) levels.[474]

The responsiveness of the HPA axis to stress is markedly reduced in late pregnancy. In rodents, this is found for psychological stressors such as exposure to a novel environment[20,482] or restraint;[19] physical stressors, including those that mimic infection, e.g., endotoxin[340] or cytokine administration;[340] and stressors that comprise both psychological and physical elements, e.g., forced swimming,[20,482,484,487] including those with ethological relevance, e.g., social stress.[488] In each case, reduced responsiveness of the HPA axis is indicated by attenuated ACTH and corticosterone secretion,[19,20,340,482,487] and where tested, is associated with reduced, or absent, stimulation of *Crh* and/or *Avp* mRNA expression in the pPVN neurons,[340,489] indicating reduced central drive of the HPA axis by the CRH/vasopressin neurons in late pregnancy. In rats reduced HPA axis responses to stress are evident from around day 15 of pregnancy and persist through pregnancy,[20] parturition,[490] and lactation, until weaning.[491] Altered responsiveness in late pregnancy involves adaptations in the anterior pituitary, hypothalamus, and higher brain centers; the mechanisms are discussed following. The mechanisms in lactation are different and are dependent on suckling.[94]

While the majority of evidence for reduced HPA axis responses in pregnancy has come from rodent studies,[94] there is also evidence for reduced HPA axis responses to stress in pregnant women.[492,493] Exogenously administered CRH fails to increase ACTH or cortisol secretion during late pregnancy in women,[494] and suppressed salivary cortisol responses are observed following exposure to the cold pressor test.[492]

Adaptations at the Anterior Pituitary in Late Pregnancy

Adaptations at the anterior pituitary contribute to reduced HPA axis responses to stress in late pregnancy in the rat. CRH is less effective in stimulating ACTH secretion and cAMP production by anterior pituitary corticotrophs in late pregnancy.[20] The reduced pituitary response to CRH is likely to be a consequence of a reduction in CRH receptor binding in the anterior pituitary in the second half of gestation.[20] Similarly, vasopressin given alone is also less effective in evoking ACTH release from anterior pituitary corticotrophs and *Avpr1b* (V1b) mRNA expression is reduced in late pregnant rats.[484] However, when administered together, ACTH responses to combined CRH and vasopressin are indistinguishable from those in virgin rats.[484] Thus any reduced responses to stress in pregnancy likely result from reduced central drive to the anterior pituitary corticotrophs.

Central Mechanisms Regulating Reduced HPA Axis Responses to Stress in Late Pregnancy

The PVN receives a rich and diverse afferent supply, including potential routes by which stressful stimuli may influence the HPA axis. The stress-processing circuit activated by a particular stressor is dependent upon the nature of the stressor. Generally stressors are divided into two categories, referred to as physical (also termed physiological or systemic) and psychological (also referred to as emotional, processive, or neurogenic).[495] Psychological stressors (e.g., restraint, novel

environment) involve sensory processing and a distinct cognitive component. They rely on the individual perceiving and appraising the situation as being stressful and are processed predominantly via rostral corticolimbic brain regions. Whereas physical stressors (e.g., immune challenge, hypoxia) are generally conceived as real physiological threats to homeostasis, which the individual need not consciously appreciate. In these situations it is advantageous to bypass cognitive processing and rapidly relay information to the PVN, thus these stressors are processed primarily through caudal brain stem regions.[496] Regardless of the stressor, they all converge, by different central pathways, on the CRH/AVP neurons in the pPVN. However, it is important to note that there are reciprocal, indirect, connections between the rostral primary stressor processing structures and the brain stem stressor processing neurons, which function to integrate HPA axis responses to stress.[496–498]

Forebrain Inputs to the HPA Axis in Late Pregnancy

The rostral networks involved in stress processing and signaling to the PVN are complex.[499] Subregions of the medial prefrontal cortex, BNST, amygdala, and hippocampus are involved in integrating stressor information and regulating HPA axis responses to acute stress. Glutamate and GABA are the key transmitters that regulate CRH neuron responses to rostral inputs,[500] however, little is understood about their regulation in response to stress in pregnancy. Studies mapping Fos protein or *c-fos* mRNA expression, as an indicator of recent activation of neurons, have reported Fos induction following acute stress is similar in several forebrain limbic structures including the hippocampus and amygdala in virgin and pregnant rats.[19,394]

Brain Stem Inputs to the HPA Axis

The brain stem catecholamine system innervates brain regions involved in regulating stress responses and plays a major role in activating the HPA axis (Figure 44.6). Brain stem A2 noradrenergic neurons located in the NTS and A1 ventrolateral medulla (VLM) project both directly and indirectly (via the parabrachial nucleus or central nucleus of the amygdala and BNST) to the medial parvocellular region of the PVN and provide excitatory input to the CRH neurons.[501,502] Neurons in the pPVN express α_1 adrenergic receptors,[503] and noradrenaline and selective α_1 agonists have been shown to excite CRH neurons[504,505] and stimulate CRH gene transcription,[505] while α_1 antagonists block HPA axis activity.[504] Furthermore, electrical stimulation of the A1 and A2 input pathways increases HPA axis activity.[504] Physical stressors, including forced swimming, and systemic administration of CCK or interleukin-1β (IL-1β; an immune challenge) exert their effects on the HPA axis by activating the A2 noradrenergic neurons in the NTS, evoking noradrenaline release from their terminals in the PVN.[506–509] Thus, neurotoxic or surgical lesions of the noradrenergic input to the PVN prevent activation of the CRH neurons in response to stressors, such as systemic immune challenge.[510]

In contrast to nonpregnant rats, HPA axis responses to forced swimming,[20,487] systemic administration of CCK[506] and IL-1β[340] are suppressed in late pregnancy, reflected by attenuated ACTH and corticosterone secretion, and reduced stimulation of *Crh* mRNA expression in the pPVN.[340] This seems to be a result of reduced activity or effectiveness of excitatory noradrenergic input to the CRH neurons in late pregnancy, since immune challenge with IL-1β[340] and forced swimming[506] fail to evoke noradrenaline release in the PVN in late pregnancy (day 21). At least in the case of IL-1β, this does not appear to be a result of impaired signaling from the periphery to the brain stem noradrenergic neurons in pregnancy, as cell bodies in the A2 region of the NTS are similarly activated (using Fos as a marker of neuronal activation) in virgin and pregnant rats.[340] In late pregnancy, mRNA expression for the $\alpha 1_A$ adrenergic receptor (*Adra1a*) is modestly decreased in the pPVN.[506] This may contribute to reduced basal HPA axis activity; however, reduced noradrenergic drive to the CRH neurons is evidently of primary importance in explaining suppressed HPA axis responses to stressors relying on the noradrenergic input to the PVN neurons and is discussed following.

Inhibitory Endogenous Opioid Mechanism in Late Pregnancy

As well as regulating prolactin and oxytocin secretion (see the sections The Prolactin System: Preparation for Lactation and Magnocellular Oxytocin Neuron System in Pregnancy), endogenous opioids have a modulatory role in HPA axis regulation (Figure 44.6). In males and nonpregnant females, opioids, such as morphine, potentiate, while naloxone suppresses HPA axis responses to stress.[511] Furthermore, naloxone treatment reduces Fos induction in CRH neurons in the pPVN following systemic IL-1β administration.[512]

However, in late pregnancy opioids switch to having a net inhibitory effect on HPA axis activity, hence pretreatment with naloxone restores ACTH responses to forced swimming,[487] IL-1β,[340] and CCK[340] in late pregnant rats, and to parturition-related stimuli.[490] Opioids exert these inhibitory actions centrally, since systemic naloxone administration before IL-1β challenge results in enhanced *Crh* mRNA expression levels in the pPVN of late pregnant rats that do not differ from those in nonpregnant rats.[340] Moreover, naloxone infused directly into the PVN restores IL-1β-evoked noradrenaline release in the PVN and the IL-1β-stimulated increase in

Crh gene transcription.[340] In this case it is presumed that naloxone blocks the inhibitory actions of opioids that act presynaptically on noradrenergic nerve terminals in the pPVN, as has been demonstrated for CCK,[513] resulting in disinhibition of the excitatory input to the CRH neurons.

Several central sources of opioids could potentially restrain the responses of CRH neurons in pregnancy. Met-enkephalin is co-expressed with CRH in pPVN neurons, but *Penka* mRNA levels are not increased here in late pregnancy.[340] Beta-endorphin cells in the arcuate nucleus project directly to the PVN,[514] and *Pomc* mRNA and β-endorphin levels in the arcuate are increased in late pregnancy.[244] However, β-endorphin is unlikely to mediate opioid actions on responses to acute stress as activation of these neurons following stress is delayed.[515] Instead, the likely source of the opioids that restrain the CRH neuronal responses in late pregnancy is the NTS neurons that project to the pPVN. NTS neurons synthesize enkephalins and dynorphins,[516,517] and mRNA expression for both *Penka* and *Oprm1* is increased in the A2 region of the NTS in late pregnancy.[340] Moreover, these neurons are rapidly activated by acute stressors, such as immune challenge.[501] Thus, in late pregnancy activation of NTS neurons by IL-1β is expected to release more enkephalin from their terminals in the PVN to act on upregulated μ-opioid receptors and presynaptically inhibit noradrenaline release, affording a mechanism through which excitatory noradrenergic drive from the brain stem to the CRH neurons in the PVN can be selectively autoinhibited in late pregnancy.

In contrast to the rat, enhanced endogenous opioid inhibition does not appear to underpin suppressed HPA axis responses to acute stress during late pregnancy in the mouse,[482] and the mechanisms involved in this species are as yet not known.

Role of Pregnancy Hormones in HPA Axis Hyporesponsiveness

In contrast to lactation where maintenance of attenuated HPA axis responses to stress relies upon the suckling stimulus provided by the pups,[518] in late pregnancy the adaptations are induced by the actions of hormones in the brain.

Role of Sex Steroids: Estrogen, Progesterone, and Allopregnanolone

During the last week of pregnancy in the rat, plasma concentrations of the female sex steroids, estradiol and progesterone, are dramatically increased,[519,520] (Figure 44.1) implicating them as candidate inducers of pregnancy-related adaptations in HPA axis responsivity. However, treatment of virgin rats with estradiol alone,[521] progesterone alone,[21] or combined estrogen and progesterone (with and without progesterone withdrawal)[521] at levels that mimic pregnancy does not result in reduced HPA axis responses to stress.[521] Thus, while these sex steroids play a role in induction of opioid inhibition over oxytocin[521] and prolactin[522] secretion during late pregnancy (see the sections The Prolactin System: Preparation for Lactation and Magnocellular Oxytocin Neuron System in Pregnancy), they are not directly involved in HPA axis hyporesponsiveness at this time.[521] The progesterone metabolite and neuroactive steroid, allopregnanolone (3α-hydroxy-5α-pregnan-20-one), does however play a crucial role.[21]

In rats, progesterone secretion by the corpora lutea is essential for establishing and maintaining pregnancy. Increased plasma and brain levels of progesterone during pregnancy are accompanied by increased levels of allopregnanolone both in the circulation and in the brain.[29] While circulating and central progesterone peaks at levels around 10 times greater than prepregnancy by day 15 of pregnancy, allopregnanolone concentrations in the brain do not peak until day 19 or 20.[29]

The majority of allopregnanolone found in the brain is likely synthesized in the periphery by the liver, ovaries, placenta, and adrenal glands,[523] however, allopregnanolone is also produced in the brain. The enzyme 5α-reductase (the rate-limiting enzyme) converts progesterone into dihydroprogesterone (DHP; 20α-hydroxy-4-pregnen-3-one), which is in turn converted into allopregnanolone by 3α-hydroxysteroid dehydrogenase (3α-HSD). Both of these enzymes are expressed in the brain by astroglia,[524] although 5α-reductase activity also occurs in neurons.[524] Thus the brain can produce neurosteroids de novo,[525] and metabolize steroids produced in the periphery into neuroactive steroids.

In late pregnancy the capacity of the brain to generate neurosteroids is increased. Activity of 5α-reductase is increased in the hypothalamus,[21] and 5α-reductase (*Srd5a1*) mRNA expression is upregulated in the NTS.[21] There is also an increase in *3α-HSD* (*Dhrs9*) mRNA expression in the PVN in late pregnancy.[21] This is expected to lead to increased allopregnanolone generation for local action on neurons.

The factor(s) responsible for regulating the expression of these enzymes in the brain in pregnancy are unclear. It has been shown that 3α-HSD activity in the hypothalamus is regulated by ovarian hormones,[526] and estrogen, but not progesterone, increases hippocampal *3α-HSD* mRNA expression.[527] There is currently no evidence to indicate that estrogen or progesterone regulates 5α-reductase expression in the brain in pregnancy, however, increased levels of prolactin may play a role.[528]

ALLOPREGNANOLONE AND HPA AXIS ACTIVITY IN PREGNANCY

In pregnancy, increased allopregnanolone levels suppress HPA axis responses to stress (Figure 44.6). Studies

have shown that blocking allopregnanolone production in pregnant rats with administration of finasteride (a 5α-reductase inhibitor that reduces brain allopregnanolone content by up to 90%)[29] substantially restores HPA axis responses to IL-1β;[21] whilst allopregnanolone administration attenuates HPA axis stress responses in nonpregnant female rats[21] and in males.[529] Any stimulatory actions of elevated estrogen levels in pregnancy on HPA axis stress responses to stress, like those reported in nonpregnant rats,[530] are presumably outweighed by the suppressive actions of allopregnanolone.

As mentioned before, progesterone is ineffective in suppressing HPA axis responses to IL-1β in nonpregnant rats. Notably, the other allopregnanolone precursor, DHP, is also ineffective,[21] which highlights the importance of upregulation of both of the allopregnanolone synthesizing enzymes in the brain in late pregnancy.

ALLOPREGNANOLONE AND ENDOGENOUS OPIOID SYSTEM INTERACTIONS IN PREGNANCY

The suppressive effect of allopregnanolone on stress-induced HPA axis activity reported in pregnancy is dependent upon the actions of endogenous opioids.[21] In virgin rats, allopregnanolone treatment induces opioid inhibition over ACTH responses to systemic IL-1β,[21] and allopregnanolone treatment upregulates *Penka* mRNA expression in the NTS of virgin rats by c. 35%, comparable to the increase reported at the end of pregnancy.[340] Furthermore, finasteride treatment reduces *Penka* mRNA expression in the NTS in late pregnant rats.[21] The mechanism by which allopregnanolone upregulates *Penka* gene expression in the NTS remains unclear, however, an interaction with $GABA_A$ receptors is a possibility,[531] as has been described for *Crh* and *Avp* mRNA expression in the PVN[532] and for oxytocin gene expression in the hypothalamus at the end of pregnancy (Figure 44.6).[531]

ALLOPREGNANOLONE AND POTENTIATION OF INHIBITORY GABA ACTIONS

As mentioned before, allopregnanolone is a potent allosteric modifier at postsynaptic $GABA_A$ receptors (including those in the PVN), where it acts to enhance the effectiveness of GABA inhibition by prolonging the opening time of chloride ion (Cl^-) channels within $GABA_A$ receptors and maintaining activity of these receptors during late pregnancy.[533–535] Parvocellular CRH neurons in the PVN are under direct inhibitory GABAergic control,[536] however, it is not known whether the effectiveness of the GABA innervation of CRH neurons is enhanced in late pregnancy, as has been described for the magnocellular oxytocin neurons (see the section Magnocellular Oxytocin Neuron System in Pregnancy).[537] Action of allopregnanolone on the $GABA_A$ receptor depends upon the subunit composition,[533] with higher efficacy at δ subunit-containing receptors than on receptors containing the γ_2 subunit.[538–540] In late pregnancy in the rat, increased central levels of allopregnanolone are associated with altered expression of $GABA_A$ receptor isoforms in the hippocampus, with an increase in the expression of the δ subunit-containing $GABA_A$-R and a decrease in those containing the γ_2 subunit.[541] The resultant increase in tonic GABAergic inhibitory transmission may be important to reduce excitability in pregnancy.

Hence, in pregnancy increased levels of allopregnanolone in the brain may act to enhance the action of GABA in the PVN or on afferent inputs to the CRH neurons to suppress HPA axis stress responses.

Role of Neuropeptide Hormones: Oxytocin and Prolactin

Oxytocin is released within the brain, including in the PVN, in response to stress,[542] indicating oxytocin may be involved in modulating stress responses. Indeed, in nonpregnant rats centrally released oxytocin reduces HPA axis responses to stress.[543–546] However, in late pregnancy, an oxytocin antagonist fails to reverse the suppressed HPA axis responses to stress,[546] indicating that endogenous intracerebral oxytocin does not maintain HPA hyporesponsiveness at this time. Similarly, intracerebral infusion of prolactin suppresses stress-induced ACTH secretion in nonpregnant female rats[547] and during lactation,[548] however, it is not known whether prolactin or placental lactogen influences HPA axis activity in pregnancy.

Glucocorticoid Negative Feedback in Pregnancy

Glucocorticoids acting via GR and MR exert negative feedback control over HPA axis activity via rapid (nongenomic signaling) and slow mechanisms (genomic).[549,550] CRH neurons in the pPVN respond to glucocorticoids by releasing eCBs, which inhibit excitatory glutamatergic synaptic inputs.[551] Moreover, glucocorticoids facilitate synaptic GABA release,[551] further enhancing inhibition of pPVN neurons. The effectiveness of these mechanisms has not been tested in pregnancy, however, as corticosterone is less effective in rapidly inhibiting ACTH secretion in late pregnant rats,[521] enhanced rapid glucocorticoid negative feedback is unlikely to be involved in reduced responsiveness of the HPA axis to stress in late pregnancy.

Nonetheless, reduced basal activity of the HPA axis in late pregnancy may involve enhanced delayed glucocorticoid negative feedback since *Nr3c1* mRNA expression (indicative of increased number of GRs) in the dentate gyrus is modestly increased on day 21 of pregnancy.[483] Furthermore, activity of the enzyme that reactivates corticosterone from inert 11-dehydrocorticosterone, 11β-hydroxysteroid dehydrogenase type-1 (11β-HSD1),

is increased in the PVN and anterior pituitary in late pregnancy[483] potentially amplifying the glucocorticoid negative feedback signal, which may contribute to the reduction in basal expression of *Crh* and *Avp* mRNA in the pPVN[483,489] and reduced *Pomc* mRNA in the pituitary[484] observed in late pregnancy.

Adrenomedullary and Sympathetic Responses to Stress in Pregnancy

In response to stress, the sympathetic and adrenomedullary systems are also activated, stimulating the release of catecholamines,[552] i.e., adrenaline from the adrenal medulla and noradrenaline from sympathetic nerve terminals. Glucocorticoids and catecholamines act together to mobilize energy stores and redistribute resources: they increase blood pressure and cardiac output and promote the delivery of substrates to tissues that are critical to the immediate defense of the animal, thus enabling them to cope with emergency situations and facilitate "fight or flight" responses.[553]

Few studies have investigated the responsiveness of the sympathetic-adrenomedullary system in pregnancy. In women, plasma noradrenaline concentrations are similarly increased in pregnant and nonpregnant participants following acute thermal stress (exposure to a heat chamber at 70 °C); however, adrenaline responses to the same stressor are suppressed in pregnant women.[554] Consistent with this is the finding in late pregnant rats of attenuated adrenaline responses, but normal noradrenaline responses to air-puff startle.[555] It is unlikely that there is a reduction in the capacity of the adrenal medulla to produce adrenaline, as *Th* (gene encoding the rate-limiting enzyme in adrenaline synthesis) mRNA expression in the adrenal medulla is increased in late pregnant rats (Douglas AJ and Gooding H, unpubl., pers. comm.). Adrenal chromaffin cells co-synthesize and co-secrete enkephalins with adrenaline;[556,557] while air-puff startle increases met-enkephalin content in the adrenal medulla of nonpregnant rats, it has no such effect in late pregnant rats (Douglas AJ, Pierzchała-Koziec K, and Russell JA, unpubl.), further supporting the prospect of reduced adrenomedullary responses to stress in pregnancy. However, the mechanisms underlying attenuated adrenaline secretory responses to stress in late pregnancy in rats and women remain to be elucidated.

Summary and Conclusions

Attenuated HPA axis responses to stress emerge in the second half of gestation in the rat. This involves adaptations at the level of the anterior pituitary and upregulation of an inhibitory endogenous opioid mechanism in the brain that is driven by increased levels of the progesterone metabolite, allopregnanolone. Progesterone treatment alone (to mimic pregnancy levels) is not sufficient to suppress HPA axis responses to stress in nonpregnant rats, indicating that an increase in the activity of 5α-reductase (which converts progesterone into allopregnanolone) in pregnancy is a prerequisite. This adaptation is expected to provide a first line of defense to protect the fetuses from any adverse programming by glucocorticoids, thus withdrawal of these mechanisms or failure to maintain them appropriately might predispose the fetuses to disease in later life.[471]

Future Perspectives

Whether the mechanisms shown to suppress HPA axis responses to the physical stressor IL-1β in late pregnancy represent a global mechanism through which HPA axis response to all types of stress are attenuated requires further investigation. The pathway by which allopregnanolone upregulates *Penka* gene expression in the NTS remains unclear, though an interaction with $GABA_A$ receptors is a possibility. Moreover, whether the effectiveness of the GABA innervation of the CRH neurons is enhanced in late pregnancy and whether this contributes to HPA axis hyporesponsiveness also remains to be elucidated. There is currently no evidence to indicate whether allopregnanolone of peripheral origin (e.g., synthesized in liver) or central origin is of greatest importance in suppressing stress-induced activity of the HPA axis in pregnancy. Increased activity of 5α-reductase in the brain in late gestation indicates that allopregnanolone generated centrally is important. However, the mechanisms that regulate 5α-reductase gene expression in the brain in pregnancy are also unclear.

MAGNOCELLULAR OXYTOCIN NEURON SYSTEM IN PREGNANCY

The most important outcomes of pregnancy for successful nurturance of the offspring are the initiation of maternal behavior and lactation. Oxytocin has important actions when released in the brain in supporting the initiation of maternal behavior. Secreted from the posterior pituitary gland by the axon terminals of magnocellular PVN and SON oxytocin neurons (Figure 44.7), oxytocin has essential actions on the mammary glands to drive transfer of milk from the mammary alveoli through the milk ducts and out from the nipples into the mouths of the suckling young. Similarly, oxytocin released into the circulation has an important ancillary role in stimulating uterine contractions during parturition to promote births. To be ready to perform these functions, oxytocin neurons themselves undergo subtle, though important, changes in pregnancy, but major changes emerge in their immediate environment in the PVN and SON, and in their

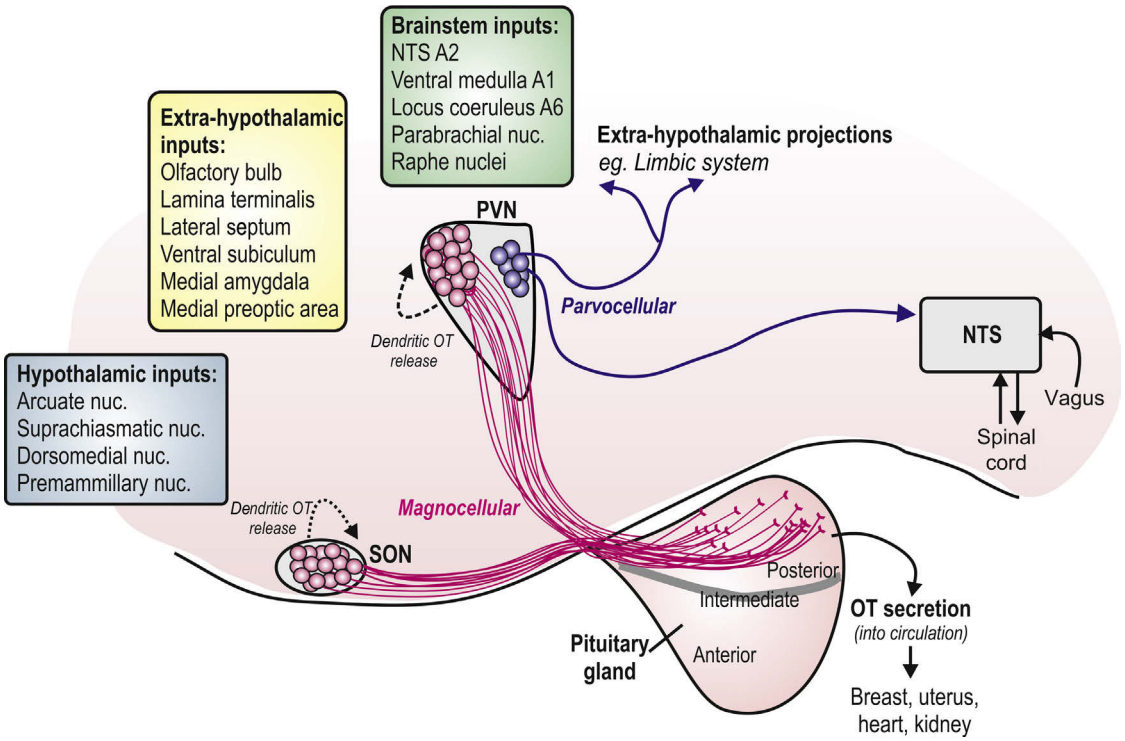

FIGURE 44.7 Oxytocin neuron projections and afferents. Magnocellular oxytocin (OT) neurons located in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) send axons to the posterior lobe of the pituitary gland. Oxytocin is secreted into the systemic circulation to act at distant organs, e.g., uterus, mammary glands. Oxytocin is also released from dendrites in the SON and PVN, where it can act locally on the oxytocin neurons themselves, but may also diffuse to influence other hypothalamic neurons, and extrahypothalamic brain regions. Parvocellular oxytocin neurons in the PVN project caudally to the nucleus tractus solitarii (NTS) and spinal cord. They also project rostrally to limbic brain regions and release oxytocin from their axon terminals. Magnocellular oxytocin neurons receive afferent inputs from hypothalamic, extrahypothalamic, and brain stem regions. Sources of input to the parvocellular oxytocin neurons include other hypothalamic nuclei (e.g., the arcuate nucleus) and the NTS.

inputs. As pregnancy progresses, oxytocin neurons show increases in auto-inhibitory mechanisms and the effectiveness of inhibitory inputs is enhanced; such changes and consequent quiescence of oxytocin neurons ensure that the oxytocin store in the posterior pituitary is enlarged and that premature activation of oxytocin neurons is prevented. The inhibitory mechanisms become less effective at the end of pregnancy, permitting their strong excitation during parturition. Oxytocin released by the dendrites of these neurons has a key role in this excitation.

Oxytocin has important actions in the brain in the context of pregnancy: oxytocin released by the dendrites of magnocellular neurons has an essential role in organizing coordinated burst firing of oxytocin neurons during parturition and suckling, which results in intermittent secretion of pulses of oxytocin; released by centrally projecting pPVN neurons (and possibly by dendrites of magnocellular oxytocin neurons, or by scarce centrally projecting axons of these neurons), oxytocin actions in rostral structures are important in initiating maternal behavior. Such actions are akin to the social affiliative actions of oxytocin, but may also involve aggressive defensive behavior. In some species, oxytocin has important peripheral actions in the control of blood volume and osmolarity (see the section Osmoregulation in Pregnancy), and is secreted in response to stressors, and in response to several metabolic signals (see the section Food Intake and Metabolism in Pregnancy).

Oxytocin and Parturition

The early finding that a posterior pituitary extract stimulated uterine contractions[558] led to the eventual identification of a nine–amino acid peptide, similar to vasopressin, that stimulates uterine contractions and milk ejections.[559] Extensive worldwide obstetric use of oxytocin for more than 50 years in humans and domestic or companion animals amply confirms the efficacy of oxytocin in initiating or promoting births. Many studies have shown that oxytocin secretion increases during parturition, as a reflex response to the passage of the fetus(es) through the birth canal (Ferguson reflex)[560] (Figure 44.8), and that the pattern of secretion is pulsatile.[561] Moreover, an oxytocin antagonist can be effective in delaying threatened preterm labor.[562,563] In rats,

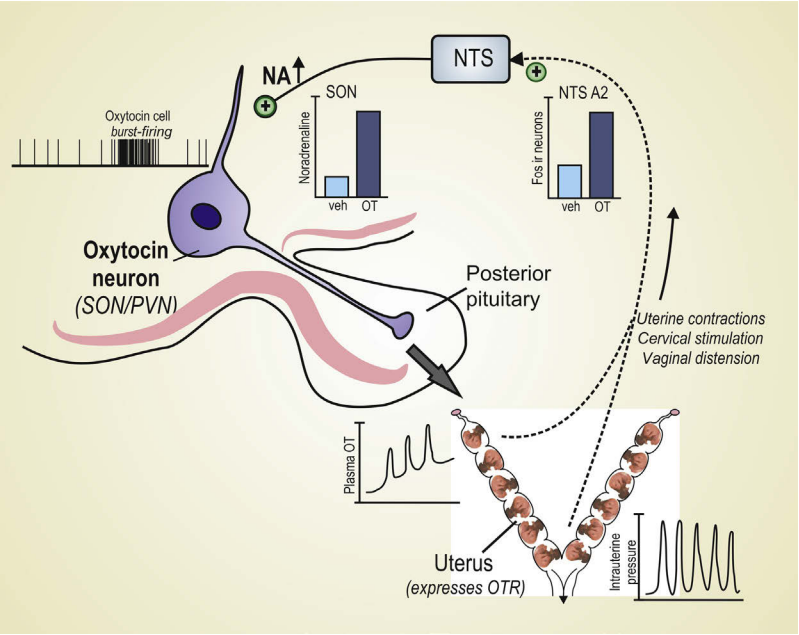

FIGURE 44.8 Oxytocin neuron activation at parturition. Coordinated burst-firing of magnocellular oxytocin neurons triggers pulsatile oxytocin (OT) secretion into the blood from the nerve terminals in the posterior pituitary. OT acting on upregulated OT receptors (OTR) in the uterus stimulates uterine contractions and increases intrauterine pressure, resulting in pup expulsion. The stretching of the birth canal activates neural pathways to noradrenergic neurons in the A2 region of the nucleus tractus solitarii (NTS). Activation of these noradrenaline (NA)-producing neurons in the NTS can be mimicked experimentally with intravenous infusion of OT pulses in day 22 pregnant rats, which causes Fos (protein product of *c-fos*, an immediate early gene and indicative of recent activation) induction in the NTS cell bodies. The noradrenergic neurons project to oxytocin neurons in the supraoptic (SON) and paraventricular (PVN) nuclei, where they release noradrenaline, which excites the OT neurons. Experimentally, in late pregnant rats intravenous OT pulses stimulate NA release in the SON. This pathway is a classic positive feedback loop, designated as the "Ferguson reflex."

inhibiting oxytocin secretion in established parturition (by opiate administration) stops births, which can be restored by oxytocin infusion,[564,565] and an oxytocin antagonist prolongs parturition.[566] However, the importance of oxytocin in parturition is challenged by the finding that mice with inactivation of the oxytocin or oxytocin receptor gene do not show deficits in parturition.[567,568] Nonetheless, magnocellular oxytocin neurons are activated during parturition in mice, an oxytocin antagonist slows parturition in mice, as does stress, and this effect is reversed by oxytocin administration.[569] Hence oxytocin, the most potent uterotonic agent, has a role in parturition in mice, but there is redundancy, and other mechanisms are also important.[570]

Oxytocin Neuron Electrical Properties

Magnocellular oxytocin neurons discharge action potentials (spikes) in a slow, irregular pattern in resting conditions (at around 4 spikes/s: in vivo, usually measured in anesthetized rats; in vitro in thin slices of the hypothalamus), which contrasts with the phasic firing pattern of vasopressin neurons.[571] When these oxytocin neurons are stimulated by, for example, increased plasma [Na^+] or intravenous (i.v.) cholecystokinin (CCK), they increase their firing rate and maintain the irregular pattern.[46,572] The irregular pattern of oxytocin neurons is a result of the balance of excitatory (e.g., glutamate, noradrenaline, and oxytocin) and inhibitory (e.g., GABA and opioids) inputs.[41,573–575]

Strikingly, during parturition and suckling magnocellular oxytocin neurons show high frequency bursts of activity (around 50–100 spikes/s for 1–2 s) a few minutes apart, and importantly these bursts happen almost simultaneously in the whole population[334,576,577] (Figure 44.8). This behavior is a result of the electrophysiological properties of the neurons and coupling among them, the actions of transmitters, and the interactions between signals emitted by the neurons, especially including oxytocin, on synapses on the dendrites.[578,579]

The Ferguson Reflex Pathway

The afferent neural pathway of the Ferguson reflex[560] has been shown to involve signals from the contracting uterus and the birth canal (uterine cervix and vagina), when it is stretched as each fetus is pushed through the canal by expulsive uterine contractions (Figure 44.8). These signals are relayed by somatic spinal afferents and by vagal afferents[580,581] to A2 noradrenergic neurons in the nucleus tractus solitarii (NTS) that project to the magnocellular oxytocin neurons in the SON and PVN. NTS neurons are also activated during parturition in mice.[569] Noradrenaline acts via α1 receptors to activate the oxytocin neurons,[573,582–584] which consequently show induction of Fos and oxytocin gene transcription[569,585,586] (Figure 44.8). In addition to release of noradrenaline in the SON, which begins an hour before the first pup is born, release of glutamate is increased transiently just before the first birth.[583] Some of the NTS neurons that project to the SON and are activated in parturition express somatostatin, which may contribute to the regulation of oxytocin neurons at this time through direct inhibitory or indirect excitatory actions.[587] The NTS neurons that are activated by vaginal distension, as in parturition, are a different population from those activated by

CCK, so there is no convergence in the pathways to oxytocin neurons of signals about parturition with satiety signals.[588] Nonetheless, CCK (and IL-1β) have been used as peripheral stimuli that act via NTS noradrenergic neurons to excite oxytocin neurons to probe adaptations in this pathway in pregnancy (discussed following).

Through this noradrenergic pathway, uterine contractions excite the electrical activity of magnocellular oxytocin neurons, increasing their continuous firing rate, hence increasing oxytocin secretion, readily measurable in blood samples.[589] Their intermittent coordinated activation during parturition, when they show brief high frequency discharge of action potentials, excites release of pulses of oxytocin from the posterior pituitary.[576] The high frequency bursts of action potentials by oxytocin neurons are only seen during parturition and, more strikingly, during suckling, when each burst is followed by secretion of a pulse of oxytocin, which causes a milk ejection, as established 40 years ago.[23] The oxytocin pulses during parturition are optimally efficient for the stimulation of uterine contractions and births, including in women.[317,590–592] However, in humans measurement is made difficult by circulating placental "oxytocinase" (placental leucine aminopeptidase; P-LAP), which has been known for over 50 years to rapidly inactivate oxytocin.[561,593] As a consequence of the intermittent pulsatile and increased continuous secretion of oxytocin, a substantial proportion of the oxytocin stored in the posterior pituitary is depleted during parturition.[594]

Provision of Oxytocin for Parturition (and Lactation)

An enlarged store of oxytocin in the posterior pituitary at the end of pregnancy is evidently the result of reduced release of oxytocin during pregnancy.

Storing Oxytocin

A result of reduced strength or activity of inputs to oxytocin neurons that mediate control of oxytocin secretion by homeostatic signals, related to osmoregulation and food intake (see the sections Osmoregulation in Pregnancy and Food Intake and Metabolism in Pregnancy), is reduced functional demand for oxytocin secretion in pregnancy. Hence, the store of oxytocin in the posterior pituitary can gradually increase through pregnancy by about 75% in rats.[393,594] This accumulation occurs without consistent findings of increased oxytocin gene expression in magnocellular neurons in pregnancy,[570,579,586,595] although such an increase does follow parturition.[586]

Sex Steroid Regulation of Oxytocin Gene Expression

The high levels of estrogen and progesterone in late pregnancy have led to studies of possible effects on oxytocin gene expression in pregnancy, but without consistent findings.[570,579] Any genomic action of estradiol could only be via ERβ,[596] as magnocellular oxytocin neurons do not express ERα.[597] Even so, the oxytocin gene promoter contains a complex composite hormone response element, with homology to a consensus estrogen response element (ERE), which shows marked species differences in binding to ERα or ERβ.[598] The response element binds orphan receptors, including steroidogenic factor-1 (SF-1), chicken ovalbumin upstream promoter transcription factor (COUP-TF),[599] and variably among species estrogen-related receptor α (ERRα),[600] and retinoic acid and thyroid hormone receptors.[598] The significance of the finding that androstanediol (3β-diol), a testosterone metabolite, briefly stimulates occupancy of the oxytocin promoter by ERβ and consequent oxytocin gene expression[601] is not clear for oxytocin synthesis during or after pregnancy. Estrogen and progesterone treatment followed by progesterone withdrawal increases *oxytocin* mRNA level in the SON and PVN, which is prevented by allopregnanolone, likely via $GABA_A$ receptors, and is consistent with no change in neuronal oxytocin gene expression in pregnancy but may explain postpartum increases.[531]

Preventing Early Oxytocin Release

Auto-Inhibition: Posterior Pituitary Opioid

Without pregnancy, μ-opioids can act on oxytocin-containing axon terminals in the posterior pituitary to inhibit voltage-gated Ca^{2+} channels (VGCC) and hence modulate bursts of oxytocin secretion.[602] However, a κ-opioid mechanism in the posterior pituitary is predominantly responsible for restraining continual oxytocin secretion, evidently mediated by a pENK-A-derived peptide produced by oxytocin neurons.[603] Importantly, this mechanism is downregulated in late pregnancy, enabling greater stimulation of oxytocin secretion by arriving actions potentials.[393]

Retrograde Signaling

Magnocellular oxytocin neurons produce and release retrograde chemical signals into the dendritic microenvironment when stimulated: these include nitric oxide (NO), eCBs, and oxytocin, which act on inputs to the neurons and inhibit transmitter release (Figure 44.9).

NEURONAL NITRIC OXIDE SYNTHASE

Oxytocin neurons express neuronal nitric oxide synthase (nNOS) and produce NO when activated.[604] In general, NO exerts its effects via cGMP-mediated increase in inhibition by GABA, and in the PVN, cGMP is expressed in oxytocin neurons and nearby GABAergic neurons and axon terminals.[604] However, in the magnocellular

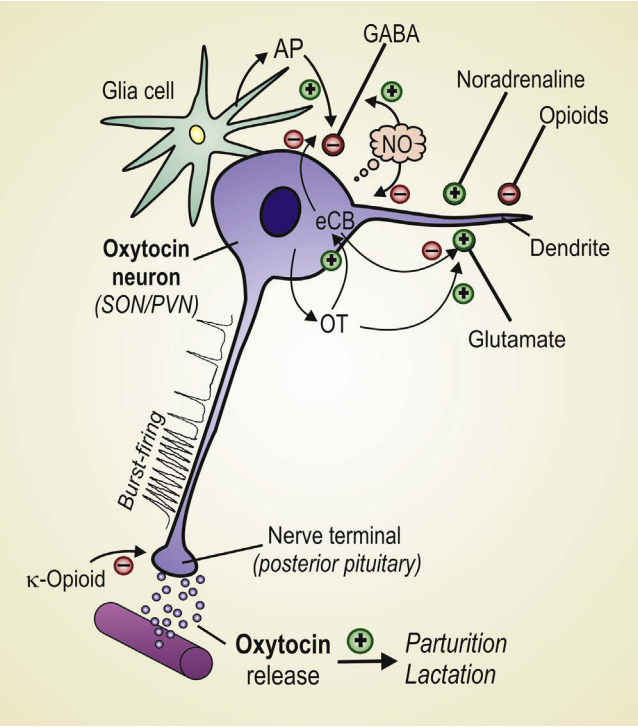

FIGURE 44.9 Microenvironment of a magnocellular oxytocin neuron: changes near the end of pregnancy. The dendrites and cell bodies of magnocellular oxytocin (OT) neurons in the supraoptic (SON) and paraventricular (PVN) nucleus receive excitatory (glutamate and noradrenaline) and inhibitory (GABA and opioids) inputs. The noradrenergic input is restrained by endogenous opioids during late pregnancy. Oxytocin is also released from the dendrites, which stimulates endocannabinoid (eCB) production by the oxytocin neurons. In turn, eCBs inhibit glutamatergic and GABA inputs to the oxytocin neurons, while oxytocin directly stimulates glutamate terminals. Allopregnanolone (AP; present in increased concentrations in pregnancy and produced by glial cells) potentiates the inhibitory actions of GABA via actions on $GABA_A$ receptors. Allopregnanolone levels decline at the end of pregnancy, and its potentiating effect on GABA inhibition of oxytocin neurons is lost. Oxytocin neurons also produce nitric oxide (NO) when activated, and NO can inhibit the oxytocin neurons both directly and by acting presynaptically on GABAergic inputs. At the end of pregnancy, the NO system in the oxytocin neurons is downregulated, thus oxytocin neurons are more responsive to excitatory inputs, and oxytocin released by the dendrites exerts a predominant control of oxytocin neuron activity. At the posterior pituitary, a κ-opioid mechanism restrains oxytocin secretion in pregnancy. This mechanism is downregulated in late pregnancy, enabling greater stimulation of oxytocin secretion by arriving actions potentials. Synchronized or burst-firing of oxytocin neurons at parturition and in lactation causes secretion of pulses of oxytocin.

oxytocin system, NO signaling has been found not to be by cGMP,[605] and electrophysiological studies show that oxytocin neurons are inhibited by NO acting directly, but also presynaptically, on GABAergic neurons (Figure 44.9).[606]

Increased capacity to produce NO in oxytocin neurons has been reported in late pregnancy, and attributed, from studies in nonpregnant rats, to stimulation by increased local oxytocin released as a result of local prolactin action.[607] However, at the end of pregnancy, *nNOS (Nos1)* mRNA expression and the inhibitory activity of the NO system on the oxytocin neurons are downregulated.[89] This attenuation of the powerful NO system restraining oxytocin neurons leaves these neurons more responsive to excitatory inputs. The importance of downregulation of NO mechanisms in oxytocin neurons is indicated by the adverse impact of i.c.v. administration of sodium nitroprusside (SNP; an NO donor) on the progress of established parturition.[608]

ENDOCANNABINOIDS AND OXYTOCIN

Release of eCBs is stimulated by increased intracellular calcium ion concentrations ($[Ca^{2+}]_i$) or depolarization, and eCBs act via G-protein-coupled CB_1 receptors. Excitatory (glutamatergic) and inhibitory (GABAergic) synapses on SON oxytocin neurons are inhibited by a CB_1 agonist, and similar actions of oxytocin are mediated by eCBs, as shown by blocking the oxytocin actions with a CB_1 antagonist.[609,610] It has been proposed that oxytocin released from dendrites will act back on OTR on the dendrites, increase $[Ca^{2+}]_i$ and hence stimulate production of eCBs, which then act on the presynaptic glutamate and GABA terminals[609,610] (Figure 44.9). Such a local cascade during parturition could serve to organize bursting activity of oxytocin neurons by quelling excitation and synchronizing inhibition. However, oxytocin also has an excitatory action on presynaptic glutamate release, which is not via eCB action.[609] Hence, if eCB production is suppressed at the end of pregnancy, dendritically released oxytocin could stimulate excitatory input. However, changes in eCB production by oxytocin neurons in pregnancy have to date not been reported.

Progesterone

In species in which progesterone in pregnancy is predominantly produced by the corpora lutea (e.g., rodents), by contrast with production by the placenta (as in women), luteolysis near term and consequent progesterone withdrawal are essential to enable uterine OTR responsiveness to oxytocin.[592,611,612] Consequently, oxytocin administration does not activate neurons in the NTS or magnocellular oxytocin neurons if progesterone withdrawal is prevented; however, eventual progesterone withdrawal after such a delay at the end of pregnancy progressively permits activation of NTS and then SON neurons.[590] Hence, progesterone has multistage actions to restrain oxytocin neurons in late pregnancy, some of which are not via progesterone receptors (PR); for example, there are no PRs in oxytocin neurons, and very few NTS neurons express PR.[124,590] Instead progesterone actions on these neurons

are mediated by allopregnanolone, a neurosteroid progesterone metabolite.

Allopregnanolone

Two types of inhibitory action by allopregnanolone have been identified: a direct action via potentiation of inhibitory actions of GABA, and an indirect action via induction of an inhibitory opioid mechanism, involving NTS A2 noradrenergic neurons (discussed following).

ALLOPREGNANOLONE AND GABA SYNAPSES

Inhibition by GABA released at synapses and acting on postsynaptic, or potentially extrasynaptic $GABA_A$, receptors[613,614] plays an important role in regulating the continuous activity of oxytocin neurons. There are GABA neurons around the magnocellular nuclei,[615,616] and the input from the lamina terminalis osmoregulatory complex includes an important GABA component.[41,46] $GABA_A$ receptors comprise five subunits, and the complex is subject to positive allosteric modulation by allopregnanolone.[613] Allopregnanolone is present in the brain in high concentrations in pregnancy,[29] and SON oxytocin neurons express predominantly α1, α2, β2, and γ2 $GABA_A$ receptor subunits,[617] of which α1 is critical for positive modulation by neurosteroid.[618] In oxytocin neurons allopregnanolone slows the closure of the $GABA_A$ receptor Cl^- channel after activation; this action is G-protein mediated, and involves protein kinase C (PKC).[619] As progesterone and consequently allopregnanolone levels fall at the end of pregnancy (in the rat), the possibility that resulting reduced GABA inhibition of oxytocin neurons may contribute to activation of oxytocin neurons at parturition has been investigated in several studies. Remarkably, the initial finding was that the potentiating effect of allopregnanolone actions on inhibition by GABA of SON oxytocin neurons is essentially lost at the end of pregnancy.[619] The initial explanation was a change in $GABA_A$ receptor subunit composition,[534] but subsequently allopregnanolone insensitivity was attributed to an action of oxytocin in the SON to activate PKC in oxytocin neurons.[400] The consequent change in balance between serine/threonine phosphatase and PKC in the neurons leads to phosphorylation of $GABA_A$ receptors, and consequent insensitivity to allopregnanolone.[400] More recently, altered α1, α2, and α3 subunit composition of clusters of $GABA_A$ receptors on SON oxytocin neurons around parturition was found, but this is not due to altered mRNA levels.[620] This change in receptor cluster composition is perhaps related to the increased numbers of GABA synapses at this time, which can be induced by exposure to oxytocin and estradiol, with action of oxytocin via OTR.[621] The observed reduced number of $GABA_A$ receptors with non-α1 subunits will reduce sensitivity to allopregnanolone, as shown in SON neurons in mice with α1 subunit gene inactivation.[400,614] Clearly, the loss of sensitivity of $GABA_A$ receptors on oxytocin neurons at the end of pregnancy will result in potential greater excitability, even if allopregnanolone level in the SON remains high, as in other brain regions.[29]

ALLOPREGNANOLONE AND ENDOGENOUS OPIOID INHIBITION

In late pregnancy magnocellular oxytocin neuron responses to i.v. CCK or IL-1β are inhibited by the endogenous opioid mechanism in the brain that emerges at this time, is induced by allopregnanolone, and also inhibits HPA axis stress responses (see the section The Hypothalamo-Pituitary–Adrenal Axis).[21,24,340,394] Both CCK and IL-1β act via noradrenergic neurons in the NTS[363] to increase the firing rate of oxytocin neurons,[24,399] in the continuous firing mode, hence increasing oxytocin secretion. CCK acts via vagal afferents and is a physiological stimulus from the small intestine signaling active digestion, acting centrally as a satiety signal, in part via oxytocin (see the section Food Intake and Metabolism in Pregnancy). IL-1β imitates immune cell activation in response to infection. It acts via interleukin receptors on endothelial cells in the dorsal medulla oblongata, initiating local prostaglandin synthesis and consequent excitation of A2 noradrenergic neurons via prostaglandin receptors.[501,510,622] Stimulation of oxytocin neurons by IL-1β is relevant to involvement of utero-cervical infection in threatened preterm labor.[623,624] Study of changes in responses of oxytocin neurons to i.v. CCK or IL-1β in pregnancy has provided information about mechanisms that may govern control of oxytocin neurons during pregnancy and parturition.

Changes in Oxytocin Neuron Responses to IL-1β in Pregnancy

In contrast with its stimulatory actions on oxytocin neurons in virgin rats, i.v. IL-1β fails to excite oxytocin neurons in late pregnancy, unless naloxone is given a few minutes before i.v. IL-1β; hence the excitation of oxytocin neurons is inhibited by an endogenous opioid mechanism at this time.[394,399] In virgin rats, i.v. IL-1β increases noradrenaline release in the PVN, as a consequence of stimulating NTS A2 neurons; but in late pregnancy, although IL-1β still activates the cell bodies of A2 neurons, noradrenaline release in the PVN is prevented by a local opioid mechanism.[340] This may involve upregulated production of presynaptic μ-opioid receptors and enkephalin in the noradrenergic input, auto-inhibiting noradrenaline release.[340] Importantly, overnight treatment of virgin rats with allopregnanolone (but not with allopregnanolone precursors, progesterone, or dihydroprogesterone) reduces oxytocin neuron responses to IL-1β as in pregnancy, and this is naloxone reversible, indicating that allopregnanolone induces opioid inhibition of oxytocin neuron responses to IL-1β.[394] Moreover, allopregnanolone treatment upregulates *Penka*

mRNA level in the NTS, a known source of enkephalin input to the PVN.[625] In late pregnant rats, blocking allopregnanolone production with finasteride reduces the upregulated expression of pENK-A mRNA.[21] Notably, expression of *5α-reductase* and *3α-HSD* mRNAs in the NTS are increased in late pregnancy, which indicates increased local allopregnanolone production.[21] Hence, increased levels of allopregnanolone in the brain, or more particularly in the NTS, in pregnancy induce the opioid inhibitory mechanism in late pregnancy that prevents oxytocin neurons from being stimulated by systemic IL-1β. The mechanisms involved in upregulation by allopregnanolone of *Penka* mRNA expression in the NTS in pregnancy are not known.

Oxytocin secretion is stimulated by other types of stressors, including forced swimming, but the response is barely altered in late pregnancy; however, the oxytocin response is much more enhanced by naloxone in late pregnant than in virgin rats, consistent with the just-discussed actions of allopregnanolone and inhibition by endogenous opioids.[487] However, estradiol and progesterone treatment for 17 days to simulate pregnancy in virgin rats leads to exaggerated oxytocin responses to forced swimming, compared with sham-treated rats, but only after opioid inhibition is removed.[487,521] Whether the sex steroid treatment leads to increased allopregnanolone production is not known.

Changes in Oxytocin Neuron Responses to CCK in Pregnancy

In late pregnancy, i.v. CCK stimulates magnocellular oxytocin neuron electrical activity and oxytocin secretion to an extent similar to that seen in virgin rats.[24] However, this conceals strong opioid inhibition of the firing rate and secretory responses in late pregnancy as these responses are much greater after naloxone, but not in virgin rats.[24] The explanation for the exaggerated responses of oxytocin neurons to i.v. CCK after naloxone seems likely to be that at the end of pregnancy, even without naloxone, CCK stimulates the release of more noradrenaline onto oxytocin neurons,[378] but the excitatory effects of this are inhibited by local opioid action. Increased noradrenaline release onto oxytocin neurons at the end of pregnancy is likely to involve the action of local oxytocin, released from the dendrites.[378,579] Moreover, noradrenaline has presynaptic excitatory actions on glutamate release onto magnocellular neurons in the SON and PVN, as well as inhibitory actions on GABA release.[626,627] In addition, glutamate as well as oxytocin and eCBs may be released by the dendrites of oxytocin neurons, as they express vesicular glutamate transporter 2 (VGLUT2).[628,629] Hence, at the end of pregnancy, in the dendritic environment of magnocellular oxytocin neurons, positive feedback mechanisms are set to be activated at the start of parturition.

Importance of Allopregnanolone and Endogenous Opioid Actions for Normal Delivery

The importance of opioid restraint of magnocellular oxytocin neurons in parturition is indicated by finding that naloxone administration in parturition increases oxytocin secretion and accelerates birth, meaning that there is insufficient time between births for full maternal behavior.[630] Conversely, the stress of moving a rat or pig from its nest during parturition leads to reduced circulating oxytocin levels and suspension of births; these effects are prevented by naloxone, and are thus due to heightened opioid inhibition of oxytocin neurons in response to stress.[630,631] The sensitivity of oxytocin neurons to inhibition by endogenous opioid inhibition is reflected in the potent inhibitory actions of μ- and κ-receptor agonists;[632] consequently, exogenous opiate drugs (e.g., morphine) strongly inhibit oxytocin neurons and births during established parturition in rats.[564] By contrast, opioid control of oxytocin neurons is not evident in pregnant mice, and stress interrupts parturition in mice by a β-adrenergic mechanism.[569]

The importance of allopregnanolone actions is indicated by finding that blocking allopregnanolone synthesis in late pregnancy by administering finasteride, which reverses the upregulation of *Penka* expression in the NTS seen in late pregnancy,[21] leads to preterm births and high neonatal mortality.[624]

Removal of Restraints and Readiness to Burst-Fire

Somato-Dendritic Release of Oxytocin in Parturition

Oxytocin released in the SON and PVN during parturition has an important role in the magnocellular nuclei in positive feedback regulation of oxytocin neuron activity, including stimulating further dendritic oxytocin release.[633] The importance of this action is revealed by the disruption of normal parturition in rats receiving an oxytocin antagonist into the SON.[633] Continuous i.c.v. infusion of an oxytocin antagonist for a week before parturition, except for the day before birth, does not affect parturition or maternal behavior but does interfere with the appropriate patterning of oxytocin secretion postpartum for a successful lactation.[634] Hence, while it is important that central oxytocin release is inhibited during pregnancy, it must be able to act at the start of parturition.

The sensitivity of oxytocin release in the PVN to an α1-adrenergic agonist increases in mid-pregnancy, evidently in preparation for stimulation of release during parturition, and subsequently during suckling in lactation, when oxytocin release in the PVN depends on local noradrenaline release, via α- and β-adrenergic receptors.[579,635] However, such increased sensitivity is not evident in late pregnancy.[579]

ENDOGENOUS OPIOID PEPTIDES

Endogenous opioid inhibition seems a likely explanation for the lack of increased sensitivity of dendritic oxytocin release to an α1-agonist in late pregnancy, as naloxone reveals that there is opioid suppression of even basal oxytocin release in the SON at this time.[24] This opioid may be either β-endorphin from arcuate POMC neurons[244] or an enkephalin produced in NTS neurons that project to the PVN and SON.[21] Notably, this important local opioid inhibition mechanism restraining somatodendritic release of oxytocin through late pregnancy is not evident during parturition, allowing enhanced oxytocin release.[636]

ALLOPREGNANOLONE

Although allopregnanolone positively modifies GABA action at $GABA_A$ receptors, in vitro allopregnanolone has a weak stimulatory effect on dendritic oxytocin release from the SON.[637] High levels of allopregnanolone in the SON in late pregnancy might thus contribute to the extracellular pool of oxytocin needed to trigger local changes in synaptic input.[579,621]

Oxytocin Receptors in the Brain in Pregnancy

Specific OTR binding sites in the PVN and SON increase in late pregnancy. In the SON (but not the PVN) this may be a result of estrogen and progesterone action, while preventing progesterone withdrawal has no effect in pregnancy.[335] Only one *Oxtr* gene has been identified,[638] and *Oxtr* mRNA expression in the SON and NTS (but not the PVN) increases in late pregnancy and is maximal at parturition.[303] Many neurons immunolabeled for OTR are activated during parturition, as shown by co-expression of Fos; this includes neurons in the SON and NTS.[303] Most NTS neurons activated in parturition and projecting to the SON express OTR, indicating that the NTS might be an additional site of positive feedback action of oxytocin.[303] Clearly, increased expression of OTR by magnocellular oxytocin neurons at the end of pregnancy is expected to increase autostimulatory actions of oxytocin in parturition.

The oxytocin receptor is a seven-transmembrane G-protein-coupled receptor that binds promiscuously to G proteins when occupied by oxytocin. Hence it directly binds G_q and $G_{i/o}$ subtypes.[639] Thus oxytocin can have diverse actions on different cell types, depending on intracellular organization of subsequent signaling pathways. In oxytocin neurons, stimulation of phospholipase C (PLC) follows G-protein binding, and triggers generation of IP3, which liberates Ca^{2+} from endoplasmic reticulum stores,[640] and this promotes exocytosis of oxytocin from dendrites.[310] In addition, synthesis of eCBs is triggered via MAP kinase or increased $[Ca^{2+}]_i$ actions.[641] Oxytocin can also modulate some K^+ channel subtypes in neurons, in a direction dependent on the type of G-protein coupled.[642] Clearly, there are multiple ways in which oxytocin may act on oxytocin neurons and their inputs (GABAergic, glutamatergic, noradrenergic, as discussed earlier) following release into the dendritic microenvironment in the PVN or SON.

Morphological Plasticity in Pregnancy

Ultrastructural studies carried out over 30 years ago showed remarkable changes in the magnocellular nuclei in the last few days of pregnancy that have been interpreted as enabling the burst-firing of oxytocin neurons during parturition and suckling post-partum. These changes include withdrawal of glial processes from between adjacent oxytocin neurons, consequent closer apposition of adjacent dendrites and cell-bodies, and evident increased numbers of synapses.[643] Such changes can be induced in virgin rats by estrogen and progesterone treatment, mimicking changes in pregnancy, provided oxytocin is given chronically by i.c.v. infusion.[644] Whether this reflects the mechanism of the changes in pregnancy is questionable as oxytocin release in the brain is not increased in pregnancy, but rather is suppressed by an opioid mechanism,[24] although remaining allopregnanolone might stimulate sufficient local oxytocin release.[637] However, preventing the neuro-glial morphological changes does not impact on parturition or burst-firing of oxytocin neurons during suckling in lactation, so the functional importance of these changes is not clear.[645]

Changes in Oxytocin Neuron Electrophysiological Properties

In late pregnancy, as in lactation, oxytocin neurons show more depolarizing afterpotentials (DAPs) after a train of action potentials, which is expected to increase excitability (see Chapter 13).[646] Oxytocin neurons also show exaggerated Ca^{2+}-dependent afterhyperpolarizations (AHPs) following a burst of action potentials, which are considered to limit the size of the burst.[579,647] Pursuing the question of the importance of oxytocin in bringing about changes in the electrical properties of oxytocin neurons in pregnancy, it has been found that central administration of an oxytocin antagonist in midpregnancy reduces the AHP changes,[648] and in vitro treatment of hypothalamic slices from late pregnant rats with oxytocin and estradiol enhances the slow AHP. However, how this, or any of these changes, occur is not clear.[579] A network of genes is proposed to underlie reorganization of SON neurons in established lactation;[649] whether this network is active in pregnancy is not yet known.

Oxytocin Neuron Burst Firing

Our understanding of the mechanisms of synchronized burst-firing by magnocellular oxytocin neurons that generate pulses of oxytocin secretion during parturition is based largely on elegant studies on the behavior of these neurons during suckling, and complemented by in vitro experiments and computer-based modeling (see Chapter 13). During parturition, burst-firing depends on stimulation by noradrenergic inputs from the birth canal as it is stretched during the passage of a fetus.[576] In lactation, burst-firing depends on the input from the nipples during suckling, although the key transmitters have not been firmly identified, noradrenergic mechanisms[650] and GABA input are important in the local control of burst-firing of oxytocin neurons.[651] Oxytocin released by the dendrites when the neurons are stimulated has an essential role: i.c.v. injection of oxytocin or infusion into the PVN or SON during suckling triggers burst-firing, and this can be stopped by an oxytocin antagonist.[334] The actions of oxytocin are through OTR located on the dendrites of the oxytocin neurons, leading to Ca^{2+} entry and mobilization from intracellular stores.[652] In lactation, oxytocin release from an enlarged pool in the SON dendrites is stimulated by glutamate (via NMDA receptors), and oxytocin released in this way acts (indirectly) as a retrograde messenger on axon terminals around oxytocin neurons in the SON to inhibit GABA release.[653,654] Oxytocin also stimulates noradrenaline release in the SON,[655] and Ca^{2+} entry into dendrites is expected to stimulate eCB production, which may then act presynaptically to modify GABAergic and glutamatergic inputs (as discussed herein).

In a cultured organotypic hypothalamic slice, oxytocin neurons show intermittent burst-firing driven by glutamate synapses and modulated by oxytocin actions.[656] In this model, low oxytocin concentrations increase GABAergic release and hence action on oxytocin neurons, while higher oxytocin concentrations inhibit GABA actions by a postreceptor mechanism.[575] The effect of the low concentration of oxytocin leads to excitation of burst-firing on recovery from GABA-induced hyperpolarization, involving Ca^{2+} influx, which likely contributes to oxytocin neuron burst-firing in vivo during suckling and parturition.[575]

OXYTOCINASE

An important factor that has recently emerged is the regulating action on the availability of extracellular oxytocin by inactivation of oxytocin by P-LAP (also referred to as oxytocinase). Immunocytochemical studies have shown expression of P-LAP in a secretory pathway in magnocellular oxytocin neurons.[656b] At the end of pregnancy P-LAP activity is reduced in the SON, which is expected to prolong the half-life of extracellular oxytocin at parturition. However, during lactation a P-LAP inhibitor, amastatin, given centrally increases the frequency of milk ejections, indicating an important role of P-LAP in curtailing oxytocin neuron bursts.[656b]

COMPUTER-BASED MODELING

This has been used to bring together in a dynamic model the factors (25 variables) that result in the burst-firing behavior of oxytocin neurons in lactation (and presumptively in parturition), in particular: (1) the electrophysiological properties of the neurons, (2) their synaptic inputs, and interactions with retrograde signals, (3) the close apposition of oxytocin neuron dendrites to each other, (4) the weak coupling that is thereby enabled: this is via oxytocin action, but not direct electrotonic coupling as previously envisaged,[657] (5) the priming of dendritic oxytocin stores for release, and (6) the positive feedback actions of oxytocin on oxytocin neurons. This model faithfully replicates the intermittent coordinated burst-firing pattern of oxytocin neurons seen during suckling.[658]

Other Consequences of Sustained Inhibition of Magnocellular Oxytocin Neurons in Pregnancy

Oxytocin has peripheral actions that promote natriuresis, and thus impact the control of extracellular fluid and volume regulation. Reduced oxytocin secretion in response to changes in blood volume and osmolarity (which without pregnancy stimulate oxytocin secretion) contribute to the changes in body fluid regulation in pregnancy (see the section Osmoregulation in Pregnancy). Furthermore, oxytocin released within the brain by magnocellular neuron dendrites may contribute to anorectic actions of oxytocin (see the section Food Intake and Metabolism in Pregnancy). Hence reduced oxytocin release in the brain in pregnancy may be an important factor in increased food intake (see the section Food Intake and Metabolism in Pregnancy).

Summary and Conclusions

Magnocellular oxytocin neurons release oxytocin from their axon terminals in the posterior pituitary in pulses during parturition and subsequently during suckling in lactation. This is the most efficient and effective way in which uterine contractions and milk ejections are driven by oxytocin; hence the magnocellular oxytocin neurons are a hub for driving these processes. Oxytocin is evidently not essential in parturition but is essential in lactation, as seen in experiments on mice with deletion of the oxytocin or oxytocin receptor genes. However, neural signals from the birth canal during parturition certainly act via spinal cord pathways and relays in the brain stem to the PVN and SON to stimulate oxytocin secretion, and oxytocin antagonists have some efficacy in delayed threatened preterm births.

The mechanism that generates pulses of oxytocin, a few minutes apart, is coordinated high-frequency action potential discharge in the whole population of magnocellular oxytocin neurons, which is only seen during parturition and lactation. This behavior is triggered by neural input but is organized by an interaction among the oxytocin neurons and with their synaptic inputs that critically depends on oxytocin released by the dendrites of these neurons. This oxytocin acts on oxytocin receptors on the neurons and increases eCB release, which then modulates excitatory and inhibitory synaptic input, having the effect of coupling the neurons. In addition, local oxytocin actions prime the dendrites for the release of more oxytocin; this positive feedback leads to an explosion of action potentials in all the neurons and secretion of an oxytocin pulse. Until late pregnancy, multiple mechanisms are active to prevent premature activation of oxytocin secretion, including nitric oxide produced by the oxytocin neurons, endogenous opioids in inputs, and allopregnanolone produced in the brain from progesterone. Oxytocinase produced by the oxytocin neurons is likely important in regulating duration of local oxytocin actions.

Future Perspectives

A computer-based model, using the known variables, replicates the coordinated burst-firing of oxytocin neurons seen in vivo. Such models enable understanding roles of new discoveries and the formulation of hypotheses. It is not clear yet which neurotransmitters in the pathway from the brain stem are most important in stimulating burst-firing during parturition; possible roles of eCBs in regulating oxytocin neurons during parturition need to be tested. Only minor changes are seen in the properties of oxytocin neurons in pregnancy, though these facilitate expression and regulation of burst-firing. The store of oxytocin in the posterior pituitary increases because of decreased release rather than upregulation of synthesis. It remains unclear how estrogen, or related steroids, might influence oxytocin gene expression.

COGNITION DURING PREGNANCY

Given the dynamic fluctuations in hormones that the maternal brain is exposed to in pregnancy (Figure 44.1), it is perhaps unsurprising that other brain systems besides those that function to ensure a successful pregnancy outcome and the expression of appropriate maternal behavior are also affected. For example, the hippocampus plays a critical role in certain aspects of learning and memory and expresses receptors for many of the hormones that show marked changes in secretion in pregnancy, such as estradiol,[659–661] progesterone,[662,663] glucocorticoids,[549] oxytocin,[664] and prolactin,[665] indicating the possibility for alterations in cognition during pregnancy. The first record in the literature reporting altered cognitive ability in pregnant women was in 1969,[666] and since then investigators have utilized rodent models to study the mechanisms involved in altered cognition during pregnancy and postpartum.

Rodent Behavioral Tests of Learning and Memory

Cognition is a complex process that comprises several components. Firstly, information must be acquired or learned, then it should be retained or stored, and lastly the stored information must be retrieved when required.

Several behavioral tests have been devised to assess cognitive performance in rodents; commonly used are the Morris water maze,[667] radial arm maze,[668] and object recognition task.[669] The Morris water maze[667] is a hippocampal-dependent task widely used to study spatial learning and memory. It comprises a large circular pool filled with water containing a hidden "escape" platform submerged just below the surface and distal cues around the pool to aid the animal's orientation. Acquisition (learning) and spatial reference memory (long-term, stable memory) can be assessed by measuring how quickly the animal learns the location of the platform over repeated trials. Alterations to the protocol can be used to assess memory retrieval and working memory (memory that is useful for a limited period of time, e.g., information unique to a specific trial). The radial arm maze[668] is another hippocampal-dependent task used to assess spatial learning and memory. In this task animals have to remember where food rewards are located: working memory is tested as the ability to remember where they just found a food reward, and reference memory is tested as the ability to remember the restricted locations of a food reward in a previous session. In the object recognition test,[669] animals are assessed on their ability to discriminate between a familiar object and a novel one. The test is based on the tendency for rodents to spend more time investigating a novel object than one previously encountered, though only if they remember the familiar one. Thus the choice to explore the novel object reflects recognition memory. The object recognition test is sometimes modified to test object place memory (the location of one of the objects, rather than the object itself is changed). Recognition tasks such as these involve processing by the hippocampus, prefrontal cortex, and/or perirhinal cortex.[670–675]

Cognition in Animals during Pregnancy

It is well established that lactation and motherhood are associated with enhanced cognition;[676,677] less is understood about cognition during pregnancy.

Behavioral Tests of Cognition during Pregnancy

Changes in cognitive performance during pregnancy depend upon the stage of pregnancy and the type of memory system being tested. During the first 14–16 days of gestation in rats, spatial memory as assessed by the Morris water maze[678] or by an object placement task[679] is enhanced compared with nonpregnant females. However, in late pregnancy (day 21) change in cognitive ability is less clear, with one study reporting that spatial memory is similar to that seen in virgin rats[680] and another indicating it is impaired,[678] using the water maze. Nevertheless, working memory or cognitive flexibility may actually be enhanced in late pregnancy[680] as pregnant rats, compared with virgin rats, display less perseveration to a previously learned location when the water maze platform is relocated.

Interestingly, prior reproductive experience influences spatial memory as rats in their second or third pregnancy perform better in a spatial memory task (days 2–17) than do primigravid rats,[681] indicating cognitive performance is further enhanced in subsequent pregnancies.

Rodent tests of cognition should however be interpreted with caution, as many tests are aversive, contain stress-invoking elements, or rely on reinforcement by rewards (commonly with food restriction). For example, the water maze, commonly used to assess spatial memory, involves forced swimming—a robust stressor in rodents.[682,683] The test relies on the animals finding the water "aversive" and/or being sufficiently "stressed" by the water in order to motivate them to escape onto the hidden platform. In nonpregnant rats, estrogen potentiates HPA axis responses to stress, while in late pregnancy the responsiveness of the HPA axis to stressful stimuli, including forced swimming, is markedly reduced (see the section The Hypothalamo-Pituitary-Adrenal Axis). Thus, findings from this type of behavioral test could be skewed by the level of stress the animal experiences. Moreover, as trials are repeated, the affective state of the animal should also be taken into account, given that repeated stressful experiences can lead to learned helplessness.[684]

Effects of Pregnancy-Related Hormones on Cognitive Performance

Changes in the hormonal milieu in pregnancy (Figure 44.1) have been proposed to underlie altered cognitive ability; however, the involvement of pregnancy-related hormones (e.g., estradiol, progesterone) on cognition has not been directly tested in pregnant animals (as blocking their action would likely compromise the pregnancy). Instead, inferences must be made from studies investigating the effects of administration of these hormones on cognitive performance in nonpregnant animals.

ESTROGENS

While there is an abundance of evidence supporting a role for estrogens in influencing cognitive performance in nonpregnant animals, findings from behavioral studies are equivocal. Such studies show that estrogens can enhance,[685–688] impair, or have no effect on learning and memory.[689–692] Critically, the effects of estrogen on cognition seem to depend upon several factors, including the nature of the task (e.g., how aversive it is), the type of memory being tested (e.g., spatial working memory, reference memory, recognition), the age[693] and sex of the animal,[694,695] the levels and pattern of estrogen exposure and when it is administered (i.e., pre- or posttraining), and the presence or absence of progesterone. For example, some studies have shown that exogenous estradiol administration to ovariectomized rats enhances memory retention in spatial memory tasks such as the Morris water maze[686,687] or open field tower task,[696] and can enhance recognition and memory retention in object recognition and object place tasks,[688] while other studies where endogenous estradiol levels are naturally higher, such as proestrus, report no effect[689] or even impaired spatial memory in the water maze.[690] Moreover, studies using the radial arm maze have found estradiol improves acquisition and working memory[697,698] but has no effect on reference memory,[697,698] whereas others have found the opposite effect, with estrogen enhancing reference memory but not working memory.[685] Discrepancies like these may result from the different doses of estrogen used (and indeed the type of estrogen used and route of administration), since low levels of estrogen have been shown to facilitate working memory,[691] while high estrogen levels impair working memory on the radial arm maze,[691,692] with similar effects on T maze alternation, a prefrontal cortex-dependent memory task.[699]

Generally, moderate levels of estrogen appear to enhance cognitive processing; however, its effects are not observed under all testing conditions. Indeed the effects of estrogens on cognition appear to be context dependent with stress/adversity and the environment playing pivotal roles in influencing estrogens actions.[696,700,701]

Long-term potentiation is a persistent enhancement in signal transmission or synaptic strength between two neurons and is considered one of the major cellular mechanisms underlying learning and memory.[702] Induction of LTP in the CA1 region of the hippocampus is augmented during proestrus (when estrogen levels are at their highest levels of the cycle) in rats.[25] In contrast, the induction of long-term depression (LTD; a persistent activity-dependent reduction in the efficacy of neuronal synapses) is severely attenuated during proestrus but not during other stages of the cycle.[25] Furthermore, induction of LTP in the hippocampus is augmented in multiparous mice compared with virgin mice.[703] Thus,

estrogen-related changes in hippocampal synaptic plasticity may be involved in altered cognition in pregnancy, however, this requires further investigation.

PROGESTERONE AND ALLOPREGNANOLONE

Although much of the research concerning the role of the female sex steroids in modulating cognition has focused on estrogens, progesterone also plays an important role. In ovariectomized females, administration of progesterone enhances object recognition in rats[704–706] and mice,[707,708] and object place recognition[709,710] and social recognition in rats.[711] Moreover, increased levels of progesterone in the hippocampus and prefrontal cortex are associated with improved Y maze performance.[712] Some of these effects of progesterone may be mediated by its metabolite, allopregnanolone.[704,705,712] Indeed, higher levels of allopregnanolone in the prefrontal cortex and hippocampus are associated with improved object recognition[712] and enhanced performance in the water maze,[704] and allopregnanolone administration improves object recognition,[705] object placement memory,[709] and performance in the water maze,[713] Y maze,[713] and in an inhibitory avoidance task.[714] Whether the fluctuating levels of progesterone and/or allopregnanolone during pregnancy (increasing progressively during early and mid-pregnancy, peaking on around day 16, before a rapid decline 2–3 days before parturition) are involved in the changes in cognition in pregnancy is unclear, though they would seem potential candidates.

OXYTOCIN

The roles of oxytocin in parturition, lactation, and maternal behavior are well established,[715] which may explain why the majority of studies investigating a role for oxytocin in cognitive processing have focused on its role in social memory and recognition. Several studies have demonstrated that central oxytocin facilitates social recognition in male rats[716,717] and mice,[568,718,719] and oxytocin also plays an important role in social recognition in female rodents.[720] Social recognition driven by olfactory cues is mediated by the actions of oxytocin and oxytocin receptors in the medial amygdala (MeA) and olfactory bulb following priming by estrogen[721] (see also Chapter 48).

The "Bruce effect" or "pregnancy block" refers to the tendency for pregnancy in female mice to be terminated by exposure to an unfamiliar male.[722] During pregnancy female wild-type mice are able to discriminate between their mate (pregnancy continues) and an unfamiliar male (pregnancy is terminated) even after a 24 h period of separation.[723] However, pregnant oxytocin knockout ($Oxt^{-/-}$) mice are unable to recognize their mate after the same period of separation, resulting in their pregnancy being terminated when exposed to the mate,[723] hence illustrating the importance of oxytocin in this olfactory memory.

The significance of oxytocin in social memory and recognition is perhaps more obviously apparent after birth. Centrally released oxytocin at parturition in ewes facilitates olfactory lamb recognition and bonding, which is essential for successful maternal behavior in this species.[26,724] In rodents, oxytocin induces LTP in the hippocampus and is critically involved in the enhanced spatial learning and memory associated with motherhood.[703] Thus central oxytocin serves to mediate social recognition before and after birth, as well as enhance spatial memory in the postpartum period, which presumably optimizes foraging for food and navigation to and from the young.

Neuroplasticity during Pregnancy and the Role of Pregnancy-Related Hormones

Estradiol and progesterone influence hippocampal structure and function. In late pregnancy, dendritic spine density in the CA1 region of the hippocampus is increased compared with virgin females (Figure 44.10).[28] This is likely a result of increased levels of circulating sex steroids, as a similar increase in spine density can be induced by a treatment regime that mimics pregnancy levels of estradiol and progesterone in ovariectomized

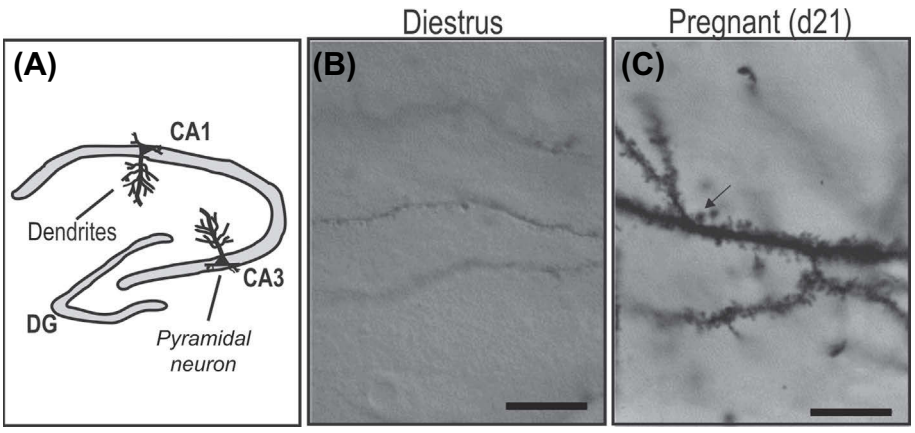

FIGURE 44.10 Dendritic spines in the hippocampus in pregnancy. (A) Diagram illustrating hippocampal pyramidal neurons and their dendritic trees. Photomicrographs of dendritic spines in the CA1 region of the dorsal hippocampus from a (B) diestrus female and (C) late pregnant (gestational day 21) rat. Scale bar = 10 μm. Note the increase in the density of spines on the dendrites (indicated by arrow) in late pregnancy compared with diestrus. As dendritic spines represent potential sites for postsynaptic input, an increase in their number is often interpreted as an increased potential for neurotransmission and information processing. *Source: Adapted from Kinsley et al.[28] with permission from Elsevier.*

virgin rats.[28] As dendritic spines represent potential sites for synaptic input, an increase in their number is often interpreted as an increased potential for neurotransmission and information processing.[725] Estradiol is a potent modulator of dendritic spine modeling, and exposure to either exogenously administered estradiol or increased endogenous levels during proestrus significantly increases the dendritic spine density in the CA1 region of the hippocampus[726–728] in rats and in nonhuman primates;[729] estradiol has been shown to enhance the number of dendritic spines in the prefrontal cortex.[730] Furthermore, in ovariectomized rats estradiol treatment increases spine density in CA1 pyramidal cells and enhances spatial memory in the Morris water maze,[731] an effect that can be augmented by progesterone.[731]

Neurogenesis during Pregnancy and the Role of Pregnancy-Related Hormones

The functional relevance of adult neurogenesis in cognition is unclear,[732] however, there is some evidence that hippocampal neurogenesis can play an important role.[733,734] Moreover, pregnancy-related hormones, in particular estradiol (see following), but also oxytocin,[735] can stimulate adult neurogenesis in the hippocampus. Changes in estradiol levels across the estrous cycle impact upon hippocampal neurogenesis in adult female rats.[736] For instance, estradiol levels in the circulation are directly correlated with cell proliferation and are inversely correlated with cell death.[736] Moreover at proestrus when endogenous estradiol reaches its highest level during the estrous cycle, rats have approximately 50% more newly proliferating cells in the dentate gyrus compared to rats at diestrus, when estradiol levels are at their lowest level,[736] an effect that can be abolished by removing the ovarian source of estradiol.[736] Estrogens evidently exert their action on neurogenesis via both ERα and ERβ, as agonists for both increase cell proliferation in the dentate gyrus.[737] In rats, ERα, ERβ, and PR agonists can enhance spatial learning,[697,709,738] while both *Erα*$^{-/-}$ (*Esr1*) and *Erβ*$^{-/-}$ (*Esr2*) mice show deficits in performing hippocampal-dependent memory tasks.[739,740] Furthermore, administration of an ERβ agonist improves cognitive performance in wild-type, but not in *Erβ*$^{-/-}$, mice.[741]

To date, few studies have directly examined the role of progesterone or allopregnanolone in neurogenesis. Nevertheless, allopregnanolone can induce a dose-dependent increase in the proliferation of neuroprogenitor cells derived from the rat hippocampus and human neural stem cells derived from the cerebral cortex in vitro.[742] In vivo, allopregnanolone promotes neurogenesis in the hippocampus and reverses cognitive deficits in a mouse model of Alzheimer's disease,[743] and furthermore has been shown to restore impairments in hippocampal neurogenesis induced by chronic isolation stress in rats.[744] Whether allopregnanolone is involved in increased neurogenesis in pregnancy remains to be elucidated.

Given these data it may be predicted that neurogenesis would be increased in pregnancy; indeed proliferation is markedly increased in the subventricular zone (SVZ) in late pregnant rats,[745] though not in the hippocampus.[745] Nonetheless, increased neurogenesis in the SVZ is also seen during pregnancy in mice,[27] an effect mediated by prolactin that results in increased incorporation of new neurons into the olfactory bulb.[27] This process is considered to play an important role in subsequent maternal behavior, given that olfactory discrimination is critical for social recognition of the offspring (see the section Maternal Behavior). Thus, in pregnancy neurogenesis may not be critical for alterations in classical hippocampal-dependent cognitive function, e.g., spatial memory, but the hormone-mediated generation of new olfactory neurons (together with the actions of oxytocin, discussed herein) may be important in enhancing social memory, especially postpartum.

Cognition in Women during Pregnancy

There is much anecdotal evidence[746,747] and data from subjective studies[748,749] indicating impaired cognitive performance during pregnancy. In a study by Poser et al.,[749] more than 80% of the women self-reported increased forgetfulness during pregnancy. In another study, two-thirds of women reported experiencing problems with one or more of the following during their pregnancy: concentration, short-term memory loss, and absent-mindedness.[748] Intriguingly, in this study more changes were reported by women who were older, married/living with partner, and had a higher level of education. However, it has been suggested that subjective memory impairments may bear little relationship to objective measures and rather may be linked with low mood (anxiety or depression)[750] or disrupted sleep.[751]

Findings employing objective measures of cognitive performance in women during pregnancy are somewhat ambiguous. Some report deficits in declarative memory (conscious recall of facts or events),[752,753] while others report no impairments in declarative memory but impaired nondeclarative memory (procedural memory, e.g., skills, habits that become automatic)[754] or impaired prospective memory (remembering to perform an intended action at the appropriate time).[755] Whereas other studies report no significant impairments in memory, despite the women in the sample self-reporting increased forgetfulness during pregnancy.[756] These studies indicate that while in women cognitive performance may be impaired during pregnancy, the effects depend upon the specific nature of the memory task employed, the stage of pregnancy, and the affective state of the woman.

Hormonal Modulators of Cognitive Function: Women

ESTROGEN

Despite estrogens having been demonstrated to have beneficial effects on certain aspects of memory in premenopausal women who have undergone ovariectomy[757] and in postmenopausal women administered estrogen replacement therapy,[758] the role of maternal estradiol levels in cognitive function during pregnancy is less clear. For example, women with higher levels of estradiol in early gestation perform less well in a verbal recall memory task in the last trimester.[759] However, other studies have found no discernible association between estradiol levels and performance scores in women in late pregnancy.[760,761]

PROGESTERONE AND ALLOPREGNANOLONE

In nonpregnant women, other sex steroid hormones known to change dramatically in pregnancy have been associated with modulating cognition. Plasma levels of the neuroactive progesterone metabolite, allopregnanolone, correlate with increased measures of confusion and delayed verbal memory recall,[762] though this may be related to the sedative effects of allopregnanolone.[763] Nevertheless, functional magnetic resonance (fMRI) studies have demonstrated that progesterone administration (possibly exerting its actions via allopregnanolone), impairs recognition memory by suppressing recruitment of brain regions such as the amygdala and fusiform gyrus during memory encoding and in the fusiform gyrus and prefrontal cortex during memory retrieval.[764] Other studies have not detected any correlation between progesterone levels and cognitive performance in gestation;[760,761] however, it is not yet known whether increased allopregnanolone level affects cognitive processing in pregnant women.

GLUCOCORTICOIDS

In the absence of pregnancy, effects of glucocorticoids on cognition are well established[765,766] and show an inverted U-shaped function,[767,768] meaning that high and low levels of glucocorticoids have detrimental effects for many cognitive processes, whereas moderate levels are associated with optimal performance. Indeed, one study of pregnant women carried out between 34 and 38 weeks of gestation reported this type of relationship between cortisol levels and performance in a verbal memory test.[761] However, another study described that lower cortisol levels between 14 and 36 weeks of gestation correlated with poorer performance in a verbal recall memory task in the last trimester.[759]

DEHYDROEPIANDROSTERONE

The evidence for effects of dehydroepiandrosterone (DHEA; a weak androgen secreted in large amounts by the adrenal) on cognitive function is inconsistent. While some studies demonstrate positive correlations between DHEA levels and cognitive function,[769,770] others indicate increased DHEA levels contribute to impaired cognitive function[771] or improve cognitive scores only in elderly patients with preexisting mild-moderate cognitive impairment[772] but are without effect in healthy older women.[773,774] Increased levels of DHEA in late pregnancy have been associated with increased performance in a line orientation task,[760] but there is little other evidence currently available.

PROLACTIN AND OXYTOCIN

Increased levels of peptide hormones have also been implicated in modulating cognition during pregnancy. Prolactin levels increase substantially in pregnancy (See the section The Prolactin System: Preparation for Lactation; Figure 44.1).[775] One study has suggested that higher prolactin levels in late gestation are detrimental to executive function.[761] However, moderate prolactin levels are optimal for performing verbal memory recall tasks in pregnancy, whereas both higher and lower levels of circulating prolactin may be detrimental.[761] Studies in women have found no correlation between cognitive performance and plasma oxytocin concentrations.[776] This is perhaps not surprising given that unlike steroid hormones, circulating oxytocin does not cross the BBB in effective concentrations, and, furthermore, any behavioral effects of oxytocin on cognitive processing are likely a result of centrally released oxytocin acting within the brain.[777]

Hippocampal Volume in Pregnant Women

Consistent with reports of impaired cognitive performance in pregnancy in women, there is a temporary decrease in the overall volume of the brain during pregnancy, which returns to prepregnancy size within 6 months postpartum.[778] It has been proposed that elevated cortisol levels may be involved.[779–781]

Summary and Conclusions

There is an apparent inconsistency in the literature when considering the effects of pregnancy on cognition between rodents and humans. The evidence from rodents predominantly indicates enhanced cognition during pregnancy. However, the human literature points toward impaired cognition in pregnancy. It seems that enhanced cognition in rodents during pregnancy ultimately serves to maximize the survival chances of the offspring. The hormones that the brain is exposed to during pregnancy prepare the animal for the challenges of motherhood. More efficient learning and enhanced memory are just one critical aspect of this. Enhanced social memory facilitates recognition of her offspring and mother–infant bonding, as well as aids

in the identification of possibly hostile conspecifics so she can defend her young. Improved spatial learning and memory will aid the efficiency of the mother's navigation to and from the nest while searching for food, thus minimizing the time she must leave the pups unattended and reducing the risk they will be harmed in her absence.

Future Perspectives

Whether the discrepancy between the rodent and the human literature is simply the result of species difference or some other underlying factor(s) requires further investigation. It may be that pregnant women should be tested using more ethologically relevant tasks, such as those more related to the pregnancy and impending motherhood (rather than intelligence quotient-, IQ-based tests), and enhanced cognitive abilities like those seen in rodents may be revealed. In support of this suggestion, pregnant women (at 22–33 weeks gestation) have been shown to have heightened attention scores in response to danger cues compared with nonpregnant controls.[782]

MATERNAL BEHAVIOR

Maternal behavior in mammals is necessarily expressed as the first young are born. This rapid expression of maternal behavior is possible because of the predictive adaptations in the maternal brain that have been organized in late pregnancy by the actions of hormones of pregnancy. These hormones act on mainly preexisting neural circuitry that organizes interactions with other individuals and involves recognition, emotionality, and attachment.[783] The actions of hormones evidently tune and bias this circuitry to favor interactions with the newborn that provide protection and nutrition, and are obviously essential for survival of the offspring and the species. The pregnancy hormones that are involved are prolactin and placental lactogen, and the female sex steroids, estradiol and progesterone; these hormones can all enter the brain from the circulation.

However, some species or strains display maternal behavior spontaneously without pregnancy, and virgin rats will display maternal behavior if repeatedly exposed to neonates over several days; this "sensitization" is independent of ovarian or pituitary hormones.[784] Nonetheless, prolactin accelerates the expression of maternal behavior in virgin rats.[785] Moreover, a substantial proportion of pregnant rats show maternal behavior toward pups on the last day of pregnancy; hence the neural mechanisms for rapid expression of maternal behavior become active before parturition.[786] This activation depends on the prolonged actions of progesterone, estradiol, and prolactin or placental lactogen through pregnancy (Figure 44.1). Hence, rats ovariectomized and hysterectomized at day 16 of pregnancy show rapid induction of maternal behavior,[787] which can be further advanced by a single injection of estradiol.[788] Moreover, treating virgin rats with sequential progesterone (days 1–11), estradiol (days 11–17), and then prolactin to simulate pregnancy changes leads to rapid expression of maternal behavior on exposure to young; progesterone withdrawal is important, as is the presence of prolactin.[32,34,520] Given in combination with progesterone and estrogen, i.c.v. prolactin (days 11–13), which is not effective alone, induces rapid expression of maternal behavior.[34]

Brain mechanisms that organize maternal behavior have been studied in these models as well as in normal pregnancy. Brain circuitry involved in maternal behavior includes the olfactory system, the mPOA, and the mesolimbic dopaminergic reward circuitry. Within the brain, actions of centrally released oxytocin are important, as are dopamine and opioid peptides (Figure 44.11).

Mammalian maternal behavior comprises several distinct components, with species variability in their relative importance. These are: nest building, and maintenance thereafter; immediate care of the young at birth (licking, cleaning, placentophagy); recognition and bonding; nursing; and protective defensive behavior (retrieval of young, aggression against intruders or predators). Some of these behaviors begin to emerge near the end of pregnancy (nest building, aggression), and others require birth to have been completed and the presence of the young.

The expression of maternal behavior at the end of pregnancy involves suppression of the actions of inhibitory factors (especially progesterone), as well as the activation of stimulatory factors (central oxytocin, dopamine). While maternal behaviors can be induced in virgin rats by repeated exposure over several days to neonatal pups,[789] the immediate expression of maternal behavior after birth depends on changes in the maternal brain at the end of pregnancy and immediately postpartum.

There is variability in the quality of maternal behavior within a species, and understanding the neurobiological basis of this has been a recent focus of interest as quality of nurturing impacts on offspring long-term health and welfare.[790] (see also Chapter 51.)

Neural Circuitry for Maternal Behavior

Olfactory Bulbs

Olfactory input in virgin rats evidently conveys stimuli from pups that are aversive and are processed via the MeA to produce defensive responses (Figure 44.11).[791] Suppression of these responses and activation of maternal behaviors postpartum is a function of the hormonally primed mPOA, through connections to the mesolimbic

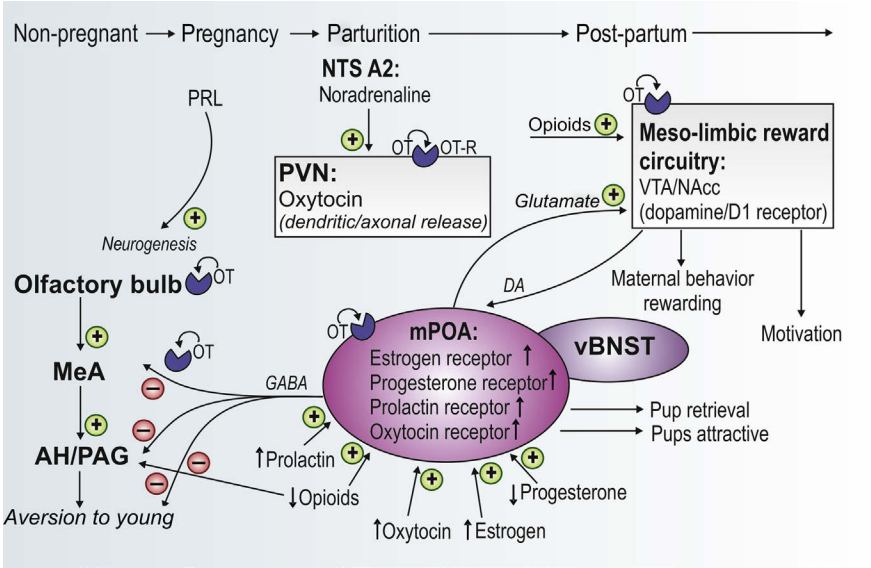

FIGURE 44.11 Neural networks organizing maternal behavior. Before pregnancy, aversion to young or disinterest is predominant, involving olfactory input to the medial amygdala (MeA) and thence to the periaqueductal gray (PAG) via the anterior hypothalamus (AH). Stimulation of neurogenesis in the olfactory bulbs by prolactin in early pregnancy is important for maternal behavior postpartum. At the end of pregnancy the medial preoptic area (mPOA) GABA output, interacting with oxytocin actions in the olfactory bulbs and amygdala, suppresses aversion to young. The mPOA is primed by actions of estrogen, progesterone, and prolactin in late pregnancy. Progesterone withdrawal and downregulation of μ-opioid action, with upregulation of oxytocin action, lead to display of maternal behavior at parturition. Central release of oxytocin stimulated by parturition, involving noradrenergic input from the nucleus tractus solitarii (NTS), promotes maternal behavior through actions at multiple sites, where oxytocin receptor expression is upregulated. mPOA projections to the meso-limbic dopaminergic (reward) circuitry stimulates motivation to perform maternally, and to provide reward via dopamine release in the NAcc. In the VTA dopamine neurons are stimulated (indirectly) by μ-opioid action, which here promotes maternal behavior, with actions on the mPOA. Activation of μ-opioid input to the PAG switches off maternal behavior. AH, anterior hypothalamus; DA, dopamine; D1, dopamine receptor; GABA, γ-aminobutyric acid; MeA, medial amygdala; mPOA, medial preoptic area; NAcc, nucleus accumbens; NTS, nucleus tractus solitarii; PAG, periaqueductal gray; OT, oxytocin; OT-R, oxytocin receptor; PVN, paraventricular nucleus; vBNST, ventral bed nucleus of stria terminalis; VTA, ventral tegmental area.

dopamine system. Hence dopamine release in this system is considered to mediate signaling of readiness to be maternal.[792]

Medial Preoptic Area

It is well-established that the mPOA has a key central role as a hub in the network that organizes and regulates the onset and maintenance of maternal behavior; the adjacent ventral BNST (vBNST) is also important (Figure 44.11).[787,793]

Estradiol, but not prolactin action specifically in the mPOA facilitates maternal behavior in the ovariectomized and hysterectomized pregnant rat model.[787] How estradiol enables neurons in the mPOA to initiate maternal behavior is not clear.[794]

PROLACTIN ACTION IN THE mPOA IN LATE PREGNANCY

As well as being essential for the preparation of the mammary glands for lactation and for milk production, prolactin has important actions in the brain that prepare for maternal behavior and reduce anxious behavior at this time.[795] It is well established that prolactin and placental lactogen actions in the brain near the end of pregnancy are important in the rapid expression of maternal behavior postpartum.[796–798] These actions are mediated by the mPOA, which expresses prolactin receptorsm,[442,799] but are also dependent on female sex steroids.[34,441,797,800–803] Hence, blocking release of prolactin from the anterior pituitary (with a D2 receptor agonist, bromocriptine, CB154) or blocking prolactin action in the brain, with a PRL-R antagonist into the mPOA, respectively, delays the normal rapid development of maternal behavior after parturition or after estradiol and progesterone treatment to simulate pregnancy.[804] More recently, studies on mice have shown prolactin actions in the brain in early pregnancy also to be important, as discussed following.[805]

PROLACTIN RECEPTORS IN THE BRAIN

PRL-R mRNA is distributed quite widely, but focally, in the brain. PRL-R_L, the active signaling form,[806] is found in the choroid plexus, anteroventral periventricular (AVPV) nucleus, arcuate, mPOA, PVN, SON, VMN, limbic regions (BNST, MeA, thalamus, central gray), and parietal cortex (during lactation). PRL-R_S is strongly

expressed in the choroid plexus and also essentially in the same regions as the long-form.[442,807,808] Notably, long-form, but not short-form *Prlr* mRNA expression in whole brain extracts is upregulated in pregnancy or by estradiol or progestogen treatment,[441,809] but the changes are localized: PRL-R_L mRNA expression is increased at the end of pregnancy (or by progesterone and estradiol treatment in virgin rats) in the mPOA and choroid plexus, but not the VMN.[442,810] In the mouse, prolactin increases pSTAT5 expression in most areas expressing PRL-R, excepting the PVN and median preoptic nucleus (MPN), indicating alternative postreceptor signaling in this region.[808]

mPOA and the Mesocorticolimbic Dopamine System

Extensive neuroanatomical studies have shown that the mPOA has connections via the lateral preoptic area with the VTA, and with the intermediate lateral septum, nucleus accumbens, olfactory circuitry, and motor outputs.[811,812] Through connections with the VTA, and hence the mesolimbic dopaminergic reward circuitry involving the nucleus accumbens, the mPOA regulates appetitive aspects of maternal behavior (Figure 44.11).[792,793] The lateral septum is important in maternal aggressive behavior, which is first seen in late pregnancy.[813,814]

mPOA projections to the mesolimbic dopamine system have been shown in a range of studies to be of key importance by evoking motivation (appetite) to behave maternally, and consummation provides reward for doing so via the nucleus accumbens (the "pleasure center").[815] For example, blocking D1, but not D2, receptors in the nucleus accumbens specifically disrupts postpartum maternal behavior.[816] Furthermore, in pregnancy-terminated rats (rats hysterectomized and ovariectomized on day 15 of pregnancy to investigate mechanisms of preparation for maternal behavior), infusion of a D1 agonist into the mPOA or nucleus accumbens facilitates rapid expression of maternal behavior.[817] The role of the mPOA is encapsulated as switching aversion or disinterest in the newborn to intense attraction.[382]

In rats, a tendency to kill young pups is suppressed near the end of pregnancy.[818] In mice, neurotoxic lesion of the central zone of the mPOA, which is crucial for maternal behavior, induces infanticidal behavior postpartum.[819] There are multiple neuronal phenotypes in this zone, and it is not clear which is most important for maternal behavior, although GABA neurons are predominant.[819] Unexpectedly, oxytocin neurons in the mPOA are not activated during maternal behavior.[819]

Ventral Tegmental Area

The VTA consists predominantly of dopaminergic neurons (the A10 group) and GABA neurons; the dopamine neurons form the mesocorticolimbic dopamine system, central to the reward circuitry in the brain. It is a focus for studies of mechanisms of normal (see the section Food Intake and Metabolism in Pregnancy), and abnormal, motivations and hence of drug addiction.[820] Activity in this system is important in the normal motivation for maternal behavior.[382] mPOA neurons project to the VTA, and VTA dopaminergic neurons project to the nucleus accumbens, medial prefrontal cortex and mPOA, where dopamine acts to promote maternal behavior, for example, in pregnancy-terminated rats.[817] The power of the VTA in promoting maternal behavior has been shown in virgin rats by chronically driving the dopamine neurons with an injection of pertussis toxin into the VTA, which then reduces latency to show maternal behavior.[821]

Prolactin and Neurogenesis in Early Pregnancy

The circadian pattern of increased prolactin secretion in early pregnancy in the rat (a diurnal and a nocturnal surge) was discussed earlier (see the section The Prolactin System: Preparation for Lactation). In mice there is only a single diurnal surge through the first 9 days of pregnancy.[822,823] In addition to an essential role of these prolactin surges in the maintenance of the corpora lutea (see the section The Prolactin System: Preparation for Lactation), studies on mice have shown that they have important actions in stimulating neurogenesis in the SVZ of the lateral ventricle, giving rise to new neurons that migrate to the olfactory bulbs,[27,795] as discussed earlier (Figure 44.11; see the section Cognition during Pregnancy). Similar increases in neurogenesis in the SVZ are seen near the end of pregnancy in rats.[745] In virgin rats induced to be maternal, a less-marked increase in neurogenesis in the SVZ is seen, but this seems unlikely to be a result of circulating prolactin action.[824]

In mice, the primary site of action of prolactin in stimulating neurogenesis in the SVZ is not clear: short-form PRL-R have been detected immunocytochemically in the dorsolateral corner of the mouse SVZ,[27] but PRL-R mRNA is not detectable in the SVZ, nor is there a pSTAT5 response to prolactin.[808] An action of growth factors released by the adjacent choroid plexus, which expresses abundant PRL-R and pSTAT5 in response to prolactin, might stimulate neurogenesis in the SVZ.[27,808]

Blocking actions of prolactin on SVZ neurogenesis pharmacologically results in disturbance of postpartum maternal behavior, such that mice are more anxious in a novel environment and show impaired maternal behavior (slow pup retrieval).[795] A similar outcome results from the suppression of prolactin secretion in early pregnancy that follows exposure to unfamiliar female mouse pheromones.[795] By contrast, exposure to male pheromones (over 2 weeks) stimulates prolactin secretion, increases neurogenesis in the SVZ, from which new neurons move to the olfactory bulbs, and eventually advances the emergence of maternal behavior in virgin and pregnant mice. These effects of exposure to male pheromones are dependent on

estradiol and progesterone.[805,825] The two critical actions of prolactin in early pregnancy to reduce anxiety postpartum, and to enable normal maternal behavior, seem to be a result of separate actions of prolactin.[823]

Oxytocin Actions Postpartum

Released centrally, oxytocin facilitates initiation of maternal behavior especially in conditions of environmental stress.[826,827] From studies of projections of oxytocin neurons, central oxytocin release, including by dendrites, changes in oxytocin receptor expression, and localized sites of administered oxytocin action, oxytocin has been found to have actions at several nodes in the networks organizing and controlling expression of maternal behavior (Figure 44.11). These include the mPOA, VTA,[36,828] nucleus accumbens,[829] and MeA,[815,830] where oxytocin acts by enhancing interactions with pups. Changes in pregnancy in oxytocin production and in oxytocin receptor expression in the brain were discussed earlier (see the section Magnocellular Oxytocin Neuron System in Pregnancy). In the present context, oxytocin receptor expression in late pregnancy is increased in the MeA, mPOA, olfactory bulbs, and VTA.[36,303,313,335]

Ventral Tegmental Area and Oxytocin

The VTA is a site of oxytocin action. mPOA neurons project to the VTA, and these include neurons expressing ER or oxytocin.[828,831] Oxytocin receptor binding in the VTA is increased at the end of pregnancy, and an oxytocin antagonist given into the VTA disrupts postpartum maternal behavior.[36] Oxytocin neurons in the pPVN as well as the mPOA project to the VTA, and the number of projecting neurons is greater in those lactating rats showing more intense pup licking and grooming behavior. Moreover, oxytocin infusion into the VTA increases dopamine release in the nucleus accumbens, and an oxytocin antagonist given into the VTA reduces dopamine released in the nucleus accumbens only in the more intensely maternal mothers.[828] Hence, oxytocin neurons mediate mPOA and pPVN modulation of intensity of maternal care via VTA dopaminergic neuron projections to the nucleus accumbens. This variability in maternal behavior is of importance in postnatal programming of the offspring.[790]

Opioids and Maternal Behavior

Administration of morphine, a μ-opioid receptor agonist, disrupts the expression of maternal behavior in ovariectomized and hysterectomized pregnant rats,[832] and in postpartum rats in established lactation, systemic morphine or i.c.v. administration of a selective μ-opioid (but not δ or κ-opioid receptor agonists) temporarily stops maternal behavior.[833,834] These effects indicate a potential inhibitory action of endogenous μ-opioids on maternal behavior. By contrast, if brain μ-opioid receptors are blocked in late pregnancy by i.c.v. β-funaltrexamine infusion, immediate postpartum maternal behavior is normal, but if pups are removed for a few days, memory for the behavior is not retained.[835,836] Furthermore, in early lactation naloxone prolongs nursing bouts,[836] indicating that endogenous opioid activation through nursing may act as a maternal reward, or satiety signal, hence ending a nursing bout. Rhesus macaques with a specific single nucleotide polymorphism (SNP) in the μ-opioid receptor-1 (*Oprm1*) gene show subtly stronger maternal attachment behavior toward their young, indicating a gain of function from this mutation, and an active endogenous μ-opioid mechanism regulating maternal behavior.[837] Explanations for these evidently conflicting findings of inhibitory and facilitatory opioid actions on maternal behavior relate to their multiple sites of action, in the mPOA, VTA, and periaqueductal gray (PAG).

Opioids and the Medial Preoptic Area

The inhibition of maternal behavior in lactating rats by morphine is accompanied by inhibition of Fos expression in the mPOA, which is reversed by naloxone pretreatment.[838] Further studies involving local application of morphine show that it acts in the mPOA.[839] Beta-endorphin content in the mPOA increases in pregnancy, evidently as a result of estrogen and progesterone action.[840] Moreover, μ-opioid receptors are expressed in the mPOA, and the level increases in pregnancy and decreases near term and after parturition, reflecting changing pregnancy hormone levels, and perhaps action of β-endorphin.[841] Consequently, endogenous μ-opioid action in the mPOA near the end of pregnancy can be expected to restrain the performance of maternal behavior, and withdrawal of this opioid inhibition at the end of pregnancy should thus contribute to the rapid expression of maternal behavior postpartum.

Opioids and the Ventral Tegmental Area

Opioid actions on maternal behavior involve the VTA, but here μ-opioid actions stimulate maternal behavior in rats primed to perform it.[842] The explanation for positive opioid actions on maternal behavior via the VTA is that VTA dopamine neurons are stimulated indirectly by μ-opioids, which act by inhibiting local GABAergic interneurons.[843] Any adaptations in pregnancy in opioid mechanisms impinging on dopamine neurons in the VTA have not been reported.

Periaqueductal Gray

This midbrain tegmentum region is important in organizing fear and defensive behaviors, in pain modulation,

and in the context of maternal behavior it is pivotal in switching between behaviors (Figure 44.11).[844] Changes in function are expected as a result of pregnancy as PAG neurons are modulated by progesterone and its neurosteroid metabolites.[845] Expression of maternal behavior requires the fear responses to pups characteristic of virgin rats to be suppressed, and this suppression is evident near the end of pregnancy.[846] Notably, maternal behavior toward pups activates GABAergic neurons in the caudal ventrolateral PAG (cPAGvl),[847] which may indicate active inhibition of fear responses to permit maternal behavior. Furthermore, the cPAG is essential for the kyphotic nursing posture, and lesion of this region increases maternal aggression.[848]

Opioids and the Periaqueductal Gray

The PAG expresses all three types of opioid receptor; changes in expression in a first pregnancy have not been reported, and there are no changes in a third pregnancy in levels of opioid receptor mRNA or protein (except for a decrease in δ-receptor protein).[849] Similarly, changes in pregnancy in opioid peptide content in the PAG have not been reported. Nonetheless there are important actions of opioids in the PAG that affect maternal behavior. The striking action of morphine in switching off maternal behavior in lactating rats is prevented by naloxone infusion into the PAG,[850] and is also prevented in rats with a previously made lesion of the rostral lateral PAG (rPAGl).[844] Moreover, given insect prey, lactating rats treated with morphine show predatory behavior, but not if the rPAGl has been lesioned; in this case maternal behavior is largely intact. Hence, switching by morphine (and presumptively endogenous μ-opioid peptides in the PAG) from maternal to predatory behavior is mediated by the rPAGl.[844] Whether this depends upon the actions of hormones during pregnancy remains to be established.

Postpartum Emotionality

It has been suggested that plasticity in processing in the olfactory bulbs as a result of stimulation of neurogenesis by prolactin in early pregnancy may be important in reducing postpartum anxiety via projections from the olfactory bulbs to the CeA, which has a key role in anxiogenesis.[805,851] Hence, postpartum mood disorders in women[852] might result from disorder of this anxiolytic mechanism, which depends on prolactin action early in pregnancy,[805] rather than being a result of loss of actions of progesterone or estrogen as otherwise supposed.[853]

Summary and Conclusions

As for lactation, changes in the maternal brain in pregnancy prepare for immediate expression of maternal behavior following birth. This behavior has multiple components and many neural circuits are involved. The changes in pregnancy are a consequence of actions of estrogen, progesterone, and prolactin or placental lactogen, especially near the end of pregnancy, when elements of maternal behavior can be elicited. Prolactin and placental lactogen readily enter the brain, and prolactin receptors are widely expressed. After birth, the prepared neural circuitry is activated by stimuli from the pups, mediated, reinforced, or regulated by actions of oxytocin, dopamine, and endogenous opioids in the brain, in the context of progesterone withdrawal. The mPOA is the essential hub in the networks that organize maternal behavior, which include the mesolimbic dopamine (reward) system, olfactory and other sensory, and locomotor systems. The hormonally prepared mPOA, by actions of prolactin and estrogen, switches aversion to the young to intense attraction, via interactions with brain stem networks. Oxytocin acts at multiple sites in the circuitry that organize maternal behavior, where oxytocin receptor expression may be upregulated in pregnancy. Similarly, endogenous opioid peptides have region-specific actions on maternal behavior, and are inhibitory in the mPOA, stimulatory via the mesolimbic dopamine system, and have actions in the PAG that are involved in switching to other behaviors.

Future Perspectives

The quality of maternal behavior has been associated with oxytocin-dopamine interactions, but it would seem appropriate to focus also on investigating possible variability in the activity of opioid mechanisms.

Postpartum disturbances in emotionality are common in women, but the mechanisms are poorly understood; a problem is the lack of validated experimental animal models.

CONCLUSION

We have summarized conclusions and questions to be asked in the future at the end of each section. Here we emphasize some common features of the adaptations in the maternal brain in pregnancy that are associated with physiological coping with pregnancy and preparations for birth and maternity.

The main theme has been that the exposure of the brain in pregnancy to hormones not experienced before (relaxin, placental lactogen), or to increased levels of familiar hormones (sex steroids: estradiol, progesterone, and its neurosteroid metabolite, allopregnanolone; peptides: prolactin, leptin) alters the settings of several neural networks in the brain. These networks regulate vital homeostatic systems (controlling hydromineral balance, appetite and metabolism, endocrine stress responses) that are adjusted to meet the physiological demands of pregnancy (expanded blood volume, energy demands).

These hormones also prepare neural systems that will be important in parturition (oxytocin secretion) or essential for successful motherhood (lactation: prolactin, oxytocin; maternal behavior: estrogen, progesterone, prolactin).

The actions of the hormones are mediated by specific receptors, which may be upregulated in pregnancy as a result of estrogen action, except for allopregnanolone, which acts as an allosteric modifier at $GABA_A$ receptors. Hence, the predominant type of change in the neural networks is at the molecular level, i.e., changes in expression of specific genes that mediate or modulate signaling. There are few indications of structural changes: an increase in neurogenesis in the SVZ (from actions of prolactin) is an example, and increased number of dendritic spines on hippocampal neurons (from actions of estradiol) is another. Nonetheless the changes in pregnancy are predominantly changes in the balance of excitatory and inhibitory signaling in the networks governing the functional output.

Focusing on the changes in signaling within the neural networks we have considered, there are noteworthy recurrences. With regard to the increased salt appetite that accompanies increased water intake and hyponatremic hypervolemia, suppression of central actions of oxytocin by inhibiting its release seems likely to be important, and this seems also important in the increased appetite of pregnancy. Furthermore, restraining release of oxytocin from the dendrites of magnocellular neurons is also important to prevent premature activation of burst-firing of oxytocin neurons that might otherwise trigger parturition. Activation of an endogenous opioid mechanism in pregnancy in the NTS that blocks signaling via the vagus to oxytocin and CRH neurons could account for such inhibition, as well as the attenuation of HPA axis stress responses. Allopregnanolone inhibits magnocellular oxytocin neurons directly, and it may have direct inhibitory actions on other neurons in the networks considered here to alter their outputs. Also, induction by allopregnanolone of the endogenous opioid mechanism in the NTS that inhibits oxytocin and CRH neurons might have wider actions. Moreover, allopregnanolone may have similar actions on opioid activation elsewhere, for example, in TIDA neurons to permit the prolactin surge at the end of pregnancy.

Opioid mechanisms are also important in increasing motivation (to eat or to behave maternally) through actions in the mesolimbic dopamine system, yet restrain the performance of maternal behavior at the end of pregnancy through actions in the mPOA. These opposing actions of opioids nonetheless imply upregulation of opioid mechanisms in pregnancy (as discussed earlier), while the opposing actions can be accounted for by similar inhibitory actions on either inhibitory or excitatory inputs in a network.

There seems to be a remarkable coherence of oxytocin actions through pregnancy, parturition, and the performance of maternal behavior. There is a similar coherence of prolactin actions on the brain, first in interacting with TIDA neurons and prolactin releasing factors to generate pulses of prolactin secretion, then to be involved in appetite regulation and preparation for maternal behavior.

The neural network regulating appetite and metabolism seems the most complex, and much research is focused on this, in the nonpregnant state. In the context of pregnancy we have described how the system may be adjusted by resistance to several inputs that normally reduce appetite and energy storage (such as leptin, CCK, and α-MSH in the brain), with increased activity of orexigenic neurons (e.g., arcuate NPY neurons, whose increased activity in pregnancy is not explained), and decreased activity of anorexigenic neurons (e.g., oxytocin neurons, perhaps restrained by an opioid mechanism, as discussed earlier).

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CHAPTER

45

Fetal Epigenetic Origins of Disease

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INTRODUCTION

In 1989 observations made by David Barker and colleagues led to an exciting new field of study associating the in utero experience with the susceptibility to disease in adulthood. They found that birth weight was inversely correlated with an individual's blood pressure in adulthood, as well as their susceptibility to the adult onset of cardiovascular disease.[1–3] Studies of individuals exposed in the womb to the Dutch Hunger Winter during World War II further supported Barker's work, revealing that famine exposure in utero has various adverse effects in adulthood.[4] These individuals show a higher risk of having glucose intolerance, obesity, and coronary heart disease.[4] While the associations between an adverse in utero experience and adult disease are strong, the question remains: How is the memory of the in utero exposure maintained until adulthood?

It is unlikely that a change in the genetic code is behind these phenotypes because some of these phenotypes are not transmitted transgenerationally. However, the contribution of fetal epigenetic modifications that may help maintain a molecular memory of an adverse in utero exposure is currently a rapidly expanding field of research. Epigenetic marks are modifiable through interaction with the environment and are altered with development.

While it has been established for more than half a century that disruptions within our genomic DNA can lead to disease, the fact that epigenetic changes can cause profound childhood and later-in-life disorders is a much more recent discovery. Genomic changes such as single nucleotide polymorphisms, copy number variations, insertions, and deletions have all been implicated in the etiology of disease. However, it is becoming increasingly evident that changes to the higher order structure of the genomic landscape, through epigenetics, also play a prevalent role in disease etiology.

Using a mouse model that carries the Agouti gene, elegant experiments have shown that epigenetic changes can lead to a disease phenotype in adulthood. Researchers discovered that the DNA methylation levels of the Agouti gene promoter, which are established in utero, can predispose the animal to obesity in adulthood. The level of DNA methylation within the Agouti promoter region in the fetus can be altered by supplementing the maternal diet with the methyl donors folic acid and vitamin B_{12}.[5] If the maternal diet does not contain the proper levels of the methyl donors, the offspring are prone to obesity in adulthood.[5–7] These data show that the DNA methylation pattern of a single locus, which is established in fetal life, can lead to adult disease. Furthermore, with proper supplementation of the maternal diet, the obesity phenotype can be avoided.

The study of imprinting disorders has also contributed to the understanding that epigenetic changes can cause profound childhood disease. For example, Prader–Willi syndrome (PWS) (discussed in detail later in the chapter) can be caused by an epimutation, where the paternal allele contains an improper maternal imprint.[8] Because this is such a severe childhood disorder, further understanding of how imprinting is established and maintained will help scientists develop treatments for these disorders with an epigenetic etiology.

In this chapter we begin by defining epigenetics and the various epigenetic modifications that have been implicated in the developmental origins of adult disease. We discuss the epigenetics of fertilization, which establishes the fetal

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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epigenome. At this crucial stage, genomic imprints are being established and protected. We discuss the mechanisms of genomic imprinting, and imprinting disorders, which can arise as a result of aberrant DNA methylation. We further summarize studies showing an association (or lack thereof) between assisted reproductive technologies (ART) and imprinting disorders. We discuss studies of maternal and paternal constraints such as diet and nutrient supplementation, which both alter the fetal epigenome and predispose the offspring to adult metabolic disease. We close the chapter with a discussion on fields of emerging influence in perinatology, which we believe will ultimately be found be crucial modifiers of the temporal and spatial establishment of the fetal epigenome.

EPIGENETICS

Every cell in our body arises from a single cell generated at conception. Each cell, with few exceptions, has the same exact genomic information. However, by the blastocyst stage, two very different cell types have already emerged. The trophoblasts that surround the blastocyst will give rise to the placenta and other extraembryonic tissues necessary to support the pregnancy. The cells of the inner cell mass within the blastocyst are embryonic stem cells (ESCs), which will give rise to the fetus. Both cell types have very different characteristics, and at this point early in gestation, cellular programming has already begun.

If the trophoblasts and ESCs contain the exact same genetic information, what distinguishes the two cell types from each other? How are they programmed to perform two completely separate tasks? The answer lies in the understanding of epigenetic regulation within the two different cell types. First described by Conrad Waddington, epigenetics is the study of alterations to the DNA that lead to changes in gene expression but does not alter the underlying DNA sequence.[9] Such changes can occur through altering the local chromatin structure. In all eukaryotic cells, DNA is packaged into a nucleoprotein structure called chromatin. Modifications that make the surrounding chromatin more compact will inhibit access of transcription factors to the DNA and can inhibit transcription. There are also modifications that can enhance access to the DNA through an opening of chromatin structure.

In this section we will describe the organization of chromatin structure within the eukaryotic genome. We will further discuss the epigenetic modifications that can occur within the chromatin that influence gene expression. Modifications to the histone proteins, methylation of the DNA, and the mechanisms of microRNA silencing will be discussed. Epigenetic modifications that are heritable through the cell cycle are summarized in Figure 45.1.

Chromatin Organization

All eukaryotes maintain their genome as a nucleoprotein complex, which consists of DNA wrapped around four histone proteins (Figure 45.2). The basic repeating unit of chromatin is the nucleosome. The central core of the nucleosome consists of two copies each of four histone proteins. The canonical nucleosome contains two copies of H3 and H4, which form a histone tetramer, as well as two histone H2A/H2B dimers. Together, the histone proteins form an octamer. Around this octamer, 147 base pairs of DNA wrap approximately 1.7 times in a left-handed superhelix to form the nucleosome.[10,11]

The crystal structure of the nucleosome provided much insight into the role of the histone proteins in chromatin organization. The crystal structure revealed that each of the four histones has a globular domain involved in dimer–tetramer interactions within the nucleosome core. However, each histone has an N-terminal domain, often referred to as the histone "tail", which extrudes from the nucleosome surface. While the histone tails do not contribute to the structure of individual nucleosomes, they are involved in organizing the structure of chromatin as a whole.[12] Besides an N-terminal tail, histones H2A and H2B contain C-terminal tails, which also extrude from the surface of the nucleosome.

Nucleosomes are separated from each other by stretches of DNA called "linker DNA". Visualized by cryo-electron microscopy, a simple nucleosomal array looks like beads on a string.[13] This is the primary structure of chromatin. In order to undergo further levels of compaction, the chromatin array employs both linker

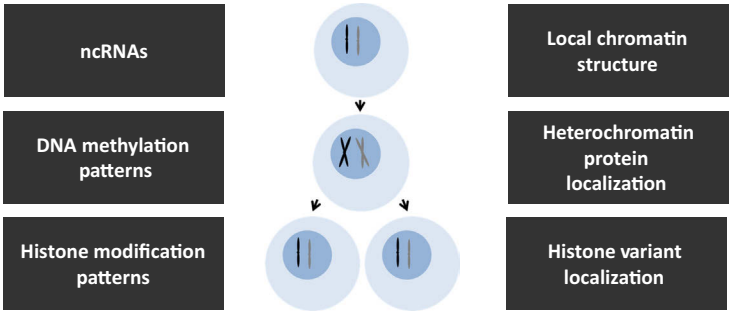

FIGURE 45.1 Epigenetic mechanisms: heritable chromatin state throughout replication and cell division. The epigenetic characteristics of a dividing cell must be passed on to the daughter cell. The mechanisms preserving the epigenetic landscape of the mother cell through cell division are unclear. However, the daughter cell requires that specific chromatin states are maintained, such as the packaging of telomeres and centromeres, in order to preserve genome stability.

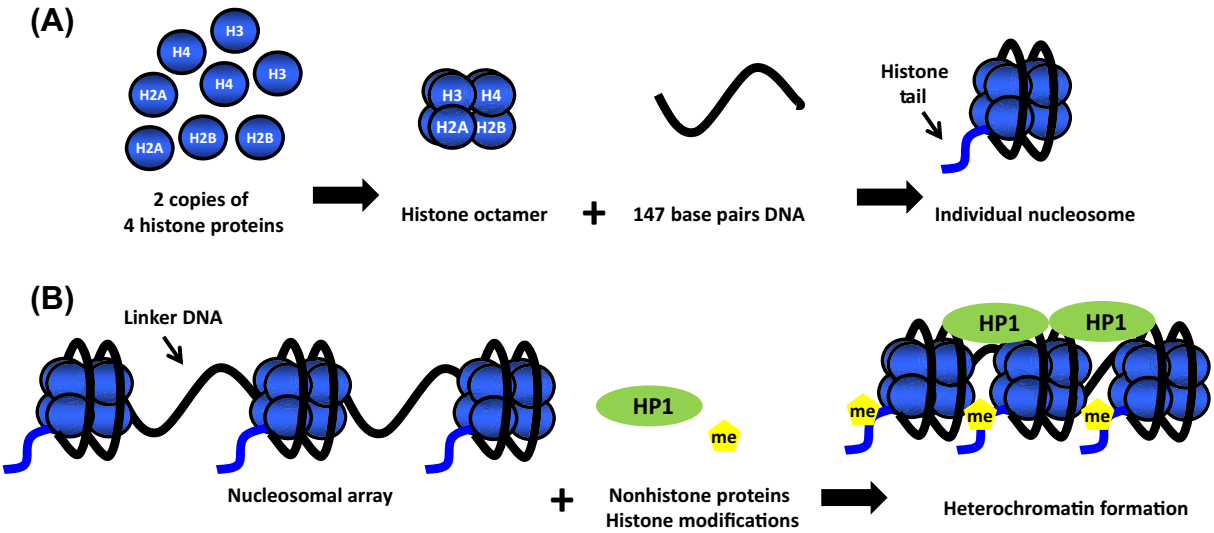

FIGURE 45.2 Chromatin organization. (A) The nucleosome is the basic repeating unit of chromatin in all eukaryotes. The nucleosome consists of two copies of four histone proteins (H3, H4, H2A, and H2B), which form the histone octamer. The nucleosome is formed by 147 base pairs of DNA that wrap around the octamer. (B) Individual nucleosomes are separated from each other by short stretches of "linker DNA" that are not wrapped around the histone octamer. In order to undergo condensation to form heterochromatin, the nucleosomal array is aided by histone modifications such as histone methylation, as well as nonhistone proteins such as HP-1.

histones, which are not part of the canonical nucleosome structure, as well as nonhistone proteins.[14] Through these interactions chromatin can form secondary and tertiary chromatin structures.

While histones H3, H4, H2A, and H2B are the four main histones based on quantity in the eukaryotic cell, there are also variants of H3 and H2A. Some variants are species specific. While crystal structures of nucleosomes with variants have been published, their exact role throughout the cell cycle and in chromatin organization still remains to be elucidated. For example, most eukaryotes have a variant of histone H3, called H3.3, which differs from H3 only by four amino acid residues.[15] This variant is synthesized throughout the cell cycle rather than only during S-phase. Histone H2A also has many characterized variants. H2A.X has a unique C-terminal tail compared with H2A. Within the H2A.X tail resides an important serine residue that is immediately phosphorylated in chromatin following a DNA double strand break.[16] The role this modified serine plays in the detection and repair of double strand breaks has been the subject of hundreds of studies.[17] Of interest to this chapter are the oocyte- and testis-specific histone variants, which will be discussed in detail in the section The Epigenetics of Fertilization.

The histone octamer makes strong contacts with the nucleosomal DNA. Therefore, specific enzymes are necessary to access the DNA, which is tightly wrapped around the octamer: ATP-dependent chromatin remodeling enzymes and histone modifying enzymes. Both are important machineries for transcription, DNA synthesis, and DNA repair. The ATP-dependent remodeling enzymes use the energy from ATP hydrolysis to disrupt histone-DNA contacts. They can do so by creating torsion of the DNA, sliding nucleosomes, and aiding histone dimer exchange.[18] ATP-dependent remodeling enzymes are involved in transcription,[19] DNA replication,[20] and DNA repair.[21]

The histone modifying enzymes add posttranslational modifications, such as phosphorylation, acetylation, methylation, and ubiquitylation to the histone proteins. Amino acids within the histone globular domains, located within the nucleosome core, are subject to modification. However, most of the histone modifications characterized thus far are found on the N- and C-terminal tails that extrude from the nucleosome surface. The importance of these histone modifications is currently an exciting field of research impacting the fields of reproduction and fertility research.

Histone Modifications

Histone modifications are known to play an important role in replication, transcription, heterochromatin formation, chromatin compaction, and DNA damage repair. As we are currently learning, the pattern of histone modifications is also important for fertility and fetal growth. Histone modifications can be altered by different environmental stimuli and may provide a "memory" of previous transcription or activation. While the role of the many histone modifications discovered thus far has not yet been established, many modifications have been associated with specific transcriptional states. For the importance of the work described in this chapter, we will

focus on histone acetylation and methylation, two modifications that have been implicated in the fetal epigenetic origins of disease. The relevant histone modifications are summarized in Table 45.1.

Many hypotheses exist as to the role of histone modifications. The phosphorylation of the C-terminal tail of histone H2A.X immediately after DNA damage is thought to recruit damage repair enzymes directly to the site of the double strand break.[22] Perhaps specific modifications aid in the recruitment of downstream effector proteins, which then aid in DNA damage repair, initiate transcription, or maintain heterochromatin structure. Histone modifications must be inherited through subsequent cell cycles to maintain the viability of the cell. For example, telomeric regions must be maintained as tightly compacted heterochromatin so that the ends of each chromosome are not recognized by the cell as DNA double strand breaks. Telomeres are enriched with histone modifications specific for heterochromatin and will retain these heterochromatic marks after a round of S-phase.[23]

Historically, specific histone modifications have been associated with specific chromatin states. There are certainly exceptions to the rule, and the role of some modifications remains elusive. Histone modifications are dynamic. The cell employs enzymes that can acetylate histones as well as enzymes that remove the acetyl group, making these marks reversible.[24] Histone lysine acetylation is a highly characterized histone modification.

Using high-throughput sequencing technology, localization of acetylated histones has been mapped throughout the genome in many cells and tissues. Using a technique called chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), the genomic location of any modification can be mapped, as long as there is an antibody that recognizes it. Lysine acetylation has traditionally been associated with transcriptionally active, open chromatin structure, called euchromatin.[25] Most studies have focused on acetylation of histone H3, specifically lysines 9 and 14 (H3K9/14ac) as well as acetylation of the four lysines within the H4 histone "tail" (K5, 8, 12, 16).[26] Acetylated histones are found near the transcription start site and within the coding region of active genes throughout the genome.[27]

Not only can histone lysines be acetylated but they can also be mono-, di-, or tri-methylated. Methylation of histone H3 lysine 4 (H3K4) is a conserved modification from yeast to man. Using ChIP-seq, the localization of each form (mon-, di-, or tri-) of methylated H3K4 has been mapped in *Saccharomyces cerevisiae* and mammalian cells,[28] and the data show that localization of trimethylation of H3K4 (H3K4me3) appears to be evolutionarily conserved. Similar to acetylated lysines, H3K4me3 maps to the promoter of active genes. However, mono- and dimethylation do not share the same pattern in yeast and mammals. Where lysine acetylation usually correlates with gene expression, lysine methylation does not show the same trend. The specific lysine in the histone tail that is methylated appears to correlate with specific transcriptional states. While H3K4me3 marks active transcription, trimethylation of lysine 9 of histone H3 (H3K9me3), as well as H3K27me3, appears to localize to silenced genes.[29]

DNA Methylation

DNA methylation is an important epigenetic modification in the regulation of gene expression and chromatin structure. It is a relatively stable modification; however, it is modifiable by environmental exposures. Global changes in DNA methylation have been implicated in cancer etiology.[29] In mammals, DNA is methylated on the C5 position of cytosine residues (5-mC). Most studies to date have studied methylation of "CpG dinucleotides", or a cytosine residue immediately 5′ to a guanine. Regions of compact, silenced heterochromatin, such as telomeres, are hypermethylated.[30] Approximately 70–80% of CpGs within the adult mammalian genome are methylated, with the exception of "CpG islands", which are largely unmethylated.[31] CpG islands are GC-rich stretches of the genome and lie within proximal promoters and enhancer regions of genes. Changes to the methylation status of CpG islands have been found to correlate with changes in gene expression. DNA methylation patterns are heritable through the mitotic cell cycle;

TABLE 45.1 Histone Modifications

Modification	Localization	References
Phosphorylated H2AX	Flanking DNA double strand breaks	14
Acetyl-H3 (H3K9,14ac) Acetyl-H4 (H4K5,8,12,16)	Promoter and gene body of actively transcribed genes	17,18
H3K4me3	Promoter and gene body of actively transcribed genes	20
H3K9me3	Promoter and gene body of repressed genes	21
H3K27me3	Promoter and gene body of repressed genes	21

the DNA methyltransferase, DNMT1, travels with the replication machinery and helps to reestablish methylation marks on the newly synthesized strand.[32]

The stability of CpG methylation was long thought to be due to the lack of a DNA demethylase.[33] The only way to "remove" the methyl mark was through active rounds of DNA synthesis without subsequent methylation of the daughter strand, which would dilute the overall levels of DNA methylation. However, this mechanism could not account for the rapid demethylation of the paternal genome following fertilization that occurs in the absence of DNA synthesis (discussed in the section The Epigenetics of Fertilization). Similarly, this mechanism could not account for changes in DNA methylation found in nondividing neurons.[34] The discovery of the modification 5-hydroxymethylcytosine (5-hmC) in the mammalian genome in 2009 has garnered attention as a possible intermediate in an active DNA demethylation process.[35,36]

Evidence for this process is still scarce but is actively being sought. TET1 is an enzyme responsible for converting 5-mCs to 5-hmCs.[36] The authors also showed that overexpression of TET1 in mammalian cells leads to a significant reduction in modified cytosines. TET1 is also important in mammalian cells for maintaining pluripotency; loss leads to increased methylation of *Nanog*, a gene required for pluripotency, as well as loss of *Nanog* gene expression.[37] TET1 overexpression has also been shown to be involved in active demethylation of a methylated plasmid.[38]

The importance of DNA methylation, and a potential process for active demethylation, makes the study of this modification of great importance in the fetal epigenetic origins of disease. In this chapter we highlight many studies where an altered in utero environment leads to changes in DNA methylation in the offspring, in tissues such as liver, pancreas, and brain. These changes in DNA methylation are associated with changes in gene expression in many cases and can lead to the adult onset of metabolic syndrome. However, if there is an active process that could remove the methylation marks, perhaps there is a chance for postnatal intervention, even if the "stage has been set" for an adult disease in utero. These are exciting fields of research that are only beginning to be understood.

Noncoding RNAs

Of all the epigenetic modifications implicated in the fetal origins of epigenetic disease, noncoding RNAs (ncRNAs) are the least understood. Because they have a large role in regulating gene transcription, they are likely to emerge as a major player in fetal epigenetics in the coming decade. Due to the prevalence of large-scale sequencing studies, it has been discovered that ncRNAs account for more RNA than protein coding RNAs in the mammalian transcriptome.[39] They can be classified as small ncRNAs (<200 bps) or large ncRNAs (lncRNAs, >200 bps).[40]

Small ncRNAs are around 20–30 nucleotides long and are associated with the Argonaute (Ago) family of proteins. They act to posttranscriptionally silence mRNA through exonucleolytic processing. Micro RNAs (miRNAs) are 20–23 nucleotides long and are processed from longer RNAs through the actions of Drosha and Dicer.[41] These miRNAs then associate with Ago and the RNA-induced silencing complex (RISC). The miRNA-containing target then finds complementary mRNA sequences and degrades the mRNA through an exonuclease activity.[40] Piwi-interacting RNAs (piRNAs) are similar to miRNAs in that they mediate silencing of mRNAs. They are generally 24–32 nucleotides long and arise from intergenic repetitive elements called piRNA clusters.[42] They do not require Drosha or Dicer, and associate with the PIWI-subfamily of the Argonaute family.[40]

LncRNAs are similar to mRNAs in that they are transcribed by RNA PolII, but they do not appear to code for functional proteins.[43] LncRNAs have different mechanisms through which they regulate transcription. Similar to short ncRNAs, they have been shown to target and degrade mRNA.[44] LncRNAs can also target chromatin modifications to specific regions of the genome to regulate transcription by acting as a scaffold.[45] They have also been hypothesized to compete with and sequester miRNA, inhibiting the repressive function of the miRNA and allowing its target to be expressed.[46]

We have highlighted some of the fundamental properties of chromatin and its organization. Not only do histone modifications, DNA methylation, and ncRNAs play a role in chromatin structure and folding, but they play a role in transcriptional regulation as well. While the DNA sequence will remain unchanged throughout development, the patterns of gene expression are highly dynamic over time. Research into the roles epigenetic marks play in altering gene expression patterns is still a relatively new field of study. Even more recent are studies of epigenetic reprogramming in the developing fetus due to varying environmental constraints. Perhaps these changes contribute to the differing susceptibility of these offspring to the adult onset of adult disease.

THE EPIGENETICS OF FERTILIZATION

Fertilization offers a unique vantage point to appreciate the cell-type specific nature of epigenetic patterning. It is well established that different cell types bear unique epigenetic signatures. Both the sperm and oocyte have very distinct chromatin packaging and epigenetic marks, specific for their purpose as gametes. However, once these two genomes combine at fertilization, a highly coordinated reorganization of both epigenomes must occur to orchestrate the precise needs of the developing embryo. In this section we address the epigenetic

changes that accompany spermatogenesis and oogenesis. We then describe the changes to both the paternal and maternal genomes upon fertilization in order to establish epigenetic marks necessary for proper development within the zygote. This process is summarized in Figure 45.3.

Sperm Epigenetics

It is not surprising that the sperm has a highly specialized and specific chromatin structure given the sperm's highly specialized role in reproduction. During meiosis, the sperm chromatin must undergo compaction to become 6–20 times denser than nucleosome-bound chromatin found in somatic cells.[8,47] It is hypothesized that this compaction allows for sperm mobility and shields the genome from DNA damage.[48] Until recently, it was believed that the paternal contribution to the developing embryo's epigenome was rather limited. However, studies over the past decade reveal that the sperm epigenome plays an important role both in male fertility as well as embryo development.

Although it has been recognized since 1971 that there are testis specific histone variants that can be incorporated into the sperm chromatin in a replication independent manner,[49] the role these variants play in higher-order chromatin structure and spermatagonia is still unknown. For example, the role of the testis-specific H3 variant, H3T, has been studied in vitro. These studies revealed that H3T can be incorporated into nucleosomes with the aid of the histone chaperone Nap2 and that an H3T-containing nucleosome is unstable compared with a canonical nucleosome.[50,51] Similarly, the testis-specific H2A variant, H2AL2, forms nucleosomes with different properties than a canonical nucleosome, including its ability to be remodeled by the ATP-dependent chromatin remodeling enzymes SWI/SNF and RSC.[52] While it appears that most of these variants disappear after fertilization,[53] the importance of the location or modification status of those variants that remain as part of the fetal epigenome has not been elucidated.

In order to achieve such a high level of compaction, sperm chromatin undergoes a complete rearrangement during meiosis. Approximately 85–95% of the sperm histone proteins are removed from the DNA and replaced by protamines.[48] Before the histones are removed they are hyperacetylated, which may ease their removal from the DNA template.[54] In mammals, transition proteins replace the histones, which are then replaced by one of two protamines, P1 and P2.[48] The ratio of P1 and P2 appears to be important for fertility and is approximately 1:1 in most fertile males.[47] The protamine sperm chromatin can now achieve a far greater level of compaction than that afforded by the canonical nucleosome. It is generally thought that the sperm genome is now quiescent, with no transcription occurring until after fertilization.[55]

Notably, even though the bulk of histones are replaced by protamines, paternal histones are retained within the sperm chromatin. It appears that their localization is programmed, and that there are distinct chromatin domains within the sperm genome.[56] Using ChIP-seq it has been found that histones with distinct modifications are located at specific regions throughout the sperm genome, which are important for embryonic development.[57] Histone modifications associated with active

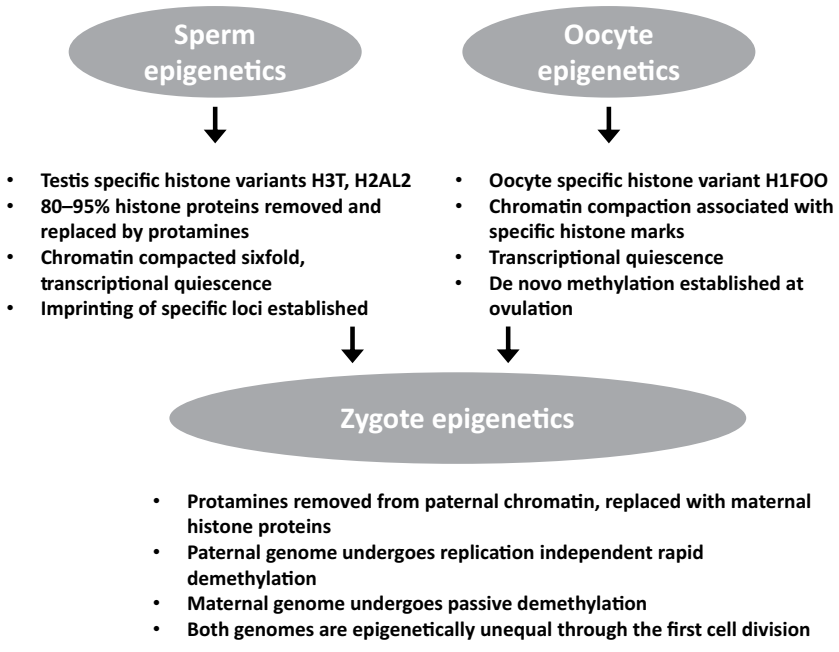

FIGURE 45.3 Epigenetics of fertilization. Both the sperm and the oocyte have distinct epigenetic landscapes that are essential for their role as gametes. However, upon fertilization both parental epigenomes must be reorganized in order to orchestrate the transcriptional program necessary for embryonic development.

chromatin (H3K4me2 and H3K4me3) are enriched at HOX gene clusters and imprinted regions, while the repressive H3K27me3 is enriched in the promoters of genes that are repressed in the developing embryo.[57] Whether these modifications help to either initiate or repress gene-specific transcription within the embryo is still unknown. Yet this data contrasts with the prevailing view that the sperm chromatin is not a player in the establishment of the embryonic epigenome. The importance of the retained histones and their location within the genome is only beginning to be elucidated. Thus, many questions still remain. What is the fate of these paternal histone proteins after fertilization? Do these histones and their associated histone code contribute to the embryonic epigenome?

Aside from the sperm histone code, the role of DNA methylation in the developing sperm is extremely important for fertility and proper embryonic development. Our understanding of DNA methylation changes during gametogenesis and embryogenesis mostly comes from studies of mouse models. In the mouse, germ cell development starts when primordial germ cells (PGCs) arise from the posterior primitive streak at embryonic day 7.5 (e7.5) and migrate to the genital ridge by e11.5. By the time the cells reach the genital ridge, an active demethylation process has removed the methylation marks of the PGCs.[58] The timing of the reestablishment of the genomic imprinting is still unknown. One study showed erasure and reestablishment of methylation before birth at a specific imprinted region in the mouse (IG-DMR), and this methylation status is maintained throughout the lifetime of the individual in the germ line.[59] Another study shows that establishment of methylation patterns of different imprinting regions occurs at different stages of germ cell development and therefore doesn't occur at a specific stage during differentiation.[60] It is generally accepted that in males imprinting is already established in gonocytes.[49]

Many studies have confirmed the importance of the paternal epigenome in fertility and proper embryo development. Using mouse models it is established that knocking out the methyltransferase DNMT1 is embryonic lethal.[61] Mice harboring a germ cell specific knockout of DNMT3a showed decreased methylation at imprinted loci, were infertile, and had decreased spermatogenesis.[62] Furthermore, treatment of mice and rats with a DNA methylation inhibitor, 5-aza-2′-deoxycytidine, led to increased preimplantation loss and decreased pregnancy rates.[63,64]

In humans, epigenetic alterations in the sperm have been shown to be associated with infertility and poor pregnancy outcomes. A study of the P1/P2 ratio in sperm used in IVF revealed that the ratio reveals a relationship with the ability of the sperm to fertilize the oocyte.[65] Another study from an IVF clinic showed that global DNA hypomethylation in sperm is associated with a poor pregnancy rate.[66] Hammoud et al. reported that sperm from infertile men had randomly distributed histone retention, rather than the programmed distribution seen in fertile men.[67]

Oocyte Epigenetics

While studies of DNA methylation patterns in sperm abound, such studies on the oocyte are scarce. This is likely due to the difficulty in obtaining enough material during the oocyte maturation process. Most of what we know about changes in DNA methylation in oogenesis in mammals has been discovered in the mouse. Traditionally, the mouse is superovulated in order to have enough eggs to study. However, such a procedure may alter the methylation patterns.[68]

Similar to the development of the sperm, in the female mouse, methylation marks within the PGCs are erased as they migrate to the genital ridge.[69] This demethylation is achieved between days 10.5 and 13.5 of gestation and is believed to occur through an active process.[70,71] However, while in the male mouse the imprinting marks have been established in the germ line before birth, in the female, de novo methylation occurs prior to ovulation over the life span of the individual.[69] As in the male germ line, establishment of an abnormal methylation pattern in the oocyte similarly disrupts embryonic development.[72]

In 1998, an oocyte-specific DNMT1 (DNMT1o) was discovered.[73] This DNA methyltransferase is abundant in the cytoplasm of the oocyte and preimplantation embryo. While it is not required for establishment of imprints in the oocyte, it is translocated from the cytoplasm to the nucleus of the eight-cell embryo, and homozygous deletion is embryonic lethal.[74,75] DNMT1o may be required for maintenance of imprints in the developing embryo. Along with DNMT1o, DNMT3A, DMNT3B, and DMNT3L are important for the correct DNA methylation patterns within the oocyte.[76] The germ line-specific DNMT3L shares a similar sequence with DNMT3A and DNMT3B but has no catalytic activity.[77] Interestingly, it is only expressed in germ cells when de novo methylation occurs; it is required to regulate DNMT3A/B activity.[78,79] Loss of DNMT3L in females prevents establishment of maternal imprints but not methylation of retrotransposons.[78]

Similar to the sperm chromatin, the oocyte chromatin also undergoes compaction, although not to the same extent as during spermatogenesis.[80] Upon compaction the oocyte achieves a transcriptionally quiescent state.[80] Fluorescence microscopy of oocyte sections from 21- to 25-day-old mice showed that oocyte maturation had varying levels of chromatin organization.[81] Specifically, in the beginning of oocyte growth the chromatin is in a decondensed state, termed "NSN", where the nucleolus

is not surrounded by chromatin.[82] As the oocyte matures, the chromatin becomes more compacted and achieves the "SN" state, where the nucleolus is surrounded by chromatin.[82] The molecular mechanisms behind the initiation of chromatin compaction and maintenance are only beginning to be discovered.

One key player in the chromatin organization of the oocyte may be the oocyte-specific linker histone, H1FOO. First described in 2001,[83] its expression is restricted to the developing oocyte.[84] While the molecular role of H1FOO is still being elucidated, preliminary results show that it may play a role in decondensing chromatin in the regions where it is bound.[85] Another protein that may play a role in chromatin condensation is nucleoplasmin 2 (Npm2). The oocytes from transgenic mice deficient for Npm2 are not able to transition from the NSN to the SN stage in oogenesis.[86] However, the $Npm2^{-/-}$ mice are still able to achieve transcriptional quiescence, even in the absence of complete chromatin condensation.[87]

The role of histone modifications and miRNA in the maintenance of proper chromatin structure during meiosis has also been evaluated. Immunohistochemistry using antibodies against acetylated histones H3 and H4 in mouse oocytes reveals that the lysines within H3 and H4 undergo almost complete deacetylation.[88] Treatment with the histone deacetylase inhibitor, trichostatin a (TSA), caused chromatin decondensation in SN oocytes.[87] Over the course of oocyte development some histone modifications remain stable (H3K9me3, H3K4me3), while others are more dynamic, changing throughout oocyte maturation (H4ac, H3R17me, H3R3me).[89] While, miRNA, siRNA, and piRNA have all been localized to the oocyte, their roles remain elusive.[90] miRNA appears to be downregulated with oocyte maturation and its activity suppressed.[91,92]

Postfertilization Epigenetics

When the two parental genomes combine during fertilization, the newly created diploid zygote must achieve totipotency within a very short amount of time. In order to do so, the epigenetic marks inherent in the sperm and egg chromatin that allowed for transcriptionally quiescent states must be reprogrammed to allow for the proper developmental genes to be expressed. The sperm chromatin, which is mostly packaged into protamines, must be reorganized. DNA methylation and histone modification patterns must be newly established. By the time the developing embryo achieves the blastocyst stage, there are two distinct lineages formed. However, in order to reach the blastocyst stage, a great deal of epigenetic reorganization must occur.

Upon fertilization, one of the first things that must occur is the replacement of the paternal protamines with maternal histones available within the cytoplasm of the oocyte. In order to achieve this, maternally derived glutathione aids in breaking the disulfide bonds between proteins.[93] Studies in mice show that by 8 h postfertilization protamines have been removed from the paternal chromatin.[94,95] With removal of protamines the sperm chromatin expands to three times the size as when it was packaged within the sperm nucleus.[48]

After the removal of protamines, the paternal chromatin goes through a round of global demethylation.[96] This global demethylation excludes centromeric heterochromatin, repeat regions, and imprinting centers.[97] It is thought to be an active process, due to the fact that it is not coupled to DNA replication, although the specifics of the process have not been completely elucidated.[98] Studies in the mouse model have revealed that the paternal genome is demethylated within 4 h following IVF.[99] Interestingly, the maternal genome appears to be protected from this active demethylation process. While the maternal genome does undergo demethylation upon fertilization, it is a passive demethylation that occurs through successive cell divisions.[100,101] It is hypothesized that remethylation is achieved, at least in part, through the activity of the oocyte-specific DNMT1o, which remains in the cytoplasm until the eight-cell stage where it translocates to the nucleus.[74,75]

Histone modifications must also be reprogrammed in the developing embryo. However, even at the two-cell stage the parental genomes are still epigenetically unequal.[89,102] In general, as the paternal genome becomes repackaged into nucleosomes, it obtains histone modifications generally associated with active chromatin, while the maternal genome appears more enriched with repressive modifications. For example, after fertilization, the paternal pronucleus is enriched with acetylated histones, while the maternal pronucleus contains less acetylated histones.[103] Sperm chromatin contains histone H4 acetylated at K8 and K12 even before decondensation is complete, suggesting these modifications are contained within the sperm chromatin.[104] As the paternal chromatin is being reestablished, histones acetylated at H4K5, 12, and 16, as well as H3K9, 14, and 18, also appear on the paternal chromatin.[104] These modifications are attributable to the histones in the cytoplasm and therefore are maternally contributed.

The paternal chromatin also lacks many histone methylation marks, although they are found in the maternal chromatin. No mono-, di-, or tri-methylation of histone H3K9 or 27 was detected in the paternal chromatin within 210 min after fertilization using IVF in the mouse.[105] These marks were visualized in the maternal chromatin. H3K4me3, a marker for transcriptionally active chromatin, is also absent from the paternal genome until 8–10 h postfertilization, and becomes indistinguishable from the levels in the maternal genome at approximately 12 h.[106]

Histone variants are also unequally distributed between the two parental genomes. While H3.1 can be found within the maternal genome, the paternal is nearly

devoid of this variant.[105] The opposite is the case with histone H3.3.[105] MacroH2A can be found in the maternal pronucleus but not the paternal.[107] However, the oocyte-specific linker histone H1FOO can be seen associated with both pronuclei. In fact, it is associated with sperm chromatin within 5 min following ICSI in the mouse.[108]

By the time the developing embryo reaches the blastocyst stage, two lineages have been formed: the inner cell mass (ICM) and the trophectoderm (TE). These lineages can be distinguished epigenetically from each other. The ICM has increased DNA and H3K9 methylation levels compared with the TE.[109] In the mouse there is also an asymmetric lineage distribution of H3K27me3 within the promoters of signaling and developmentally regulated genes.[110] It is not known why the TE is spared from the enrichment of repressive marks or how the asymmetry is delineated.[109]

The process of fertilization is unique from an epigenetic standpoint. As we have outlined, both the sperm and egg develop their own epigenetic milieu in order to function properly as gametes. Both undergo specific modifications in order to achieve a compact structure and a quiescent transcriptional state. Yet when fertilization occurs, the epigenetic state that defines each individual gamete is not ideal for a developing embryo. Both genomes undergo extensive remodeling, including global DNA demethylation and then the reestablishment of DNA methylation patterns, establishment of canonical nucleosomes throughout the paternal genome, and establishment of the zygotic histone code. The timing of many of these events has been reported, yet the mechanisms behind how and when remain largely unexplored. However, it is clear that the establishment of a proper epigenetic code in both the gametes and the embryo is essential for fertility and developmental success.

IMPRINTING

In 1984, elegant studies in the developing mouse embryo were published and first described that both parental genomes are not equivalent. Moreover, contributions from both male and female genomes are necessary for proper embryonic development. Using transplantation studies, diploid single cell mouse embryos with either two paternal pronuclei or two maternal pronuclei were created.[111] Neither was able to complete development. Surani et al. performed similar experiments, which yielded the same results; an oocyte with two maternal pronuclei cannot develop.[112] They concluded that parental imprinting, specific for the male and female genomes, is necessary for normal development. In this section we define imprinting and elaborate on the epigenetic mechanisms that maintain parent-of-origin expression. Table 45.2 summarizes the imprinted genes we discuss in this section and their associated diseases.

Mechanisms of Imprinting

Genomic imprinting ultimately results in parent-of-origin-specific, monoallelic expression of a gene. Intuitively, this seems as though it could be deleterious to development. Diploidy allows for two copies of the same gene to be expressed; a mutation in one allele may be rescued by the second fully functioning copy present in the diploid individual. Why eutherian mammals have developed a subset of genes that are regulated by genomic imprinting remains a mystery. One hypothesis, the "parental conflict theory", postulates that imprinting is a parental "tug of war" for resources.[144] In general, with imprinting genes, those that are paternally expressed (and maternally repressed) drive fetal growth, while those that are maternally expressed (and paternally repressed) moderate fetal growth by limiting maternal resources to the fetus.[113,144]

While the total number of imprinted genes is not yet known, there are 100 imprinted genes characterized in mice, and more than 50 of these maintain the imprinted status in humans.[114] Imprinting can be ubiquitous, meaning that the parent-of-origin expression is the same in all tissues. It can also be tissue specific. For example, *Mash2*, *Obph1*, and *Tssc4* are monoallelically expressed

TABLE 45.2 Imprinted Genes and Disease Associations

Gene(s)	Chromosome	Imprinted Tissues	Disease	References
H19-Igf2	11p15		SRS, BWS	110,113–124
Grb10	7p12	Most	SRS, BWS	109,125–128
Ddc	7p12	Heart	Bipolar affective disorder	111,129
Igf2r, Slc22a3, Slc22a2, Airn	6q26	Placenta	N/A	130,131
UBE3A	15q11.2	Brain	AS	132–135
SNURF-SNURPN	15q11	Brain	PWS, AS	136–139
PLAGL1, HYMA1	6q24	Fetal tissues	TNDM1	140–143

in the placenta but biallelically in other tissues.[115] *Igf2* is monoallelically expressed in almost all tissues but shows biallelic expression in the choroid plexus and leptomeninges.[116] The *Grb10* gene is paternally expressed in the brain but maternally in other tissues.[115] To date, there is only one gene where imprinting is not localized to the brain or extraembryonic tissues. *Ddc* is imprinted only in the heart.[117] The mechanisms governing the tissue-specific nature of expression are still unknown.

Most imprinted gene clusters are located within 1 MB of each other, however, imprinted genes can also be found as pairs or singletons throughout the genome.[118] Expression of imprinted genes within each cluster is under the control of an imprinting control region (ICR), also known as an imprinting center (IC) or imprinting control element (ICE).[119] DNA methylation appears to be an essential epigenetic mark for maintenance of parent-of-origin expression, although histone modifications have also been shown to play a role.[119] In fact, allele-specific methylation has been described at all known ICRs.[119]

Insulator Model of Imprinting Control

Study of the *H19-Igf2* imprinted genes has led to the most detailed mechanistic understanding of the insulator model of maintaining parent-of-origin monoallelic expression. *H19* is a noncoding RNA, expressed from the maternal allele, and is required to moderate fetal and placental growth.[120] *Igf2* is paternally expressed and is required to promote fetal and placental growth.[121] Both genes use the same enhancers for expression.[122] The differentially methylated region (DMR), which controls expression of these two genes, lies between them and spans approximately 2.4 kb.[123] This DMR serves as the ICR.

In general, there are two types of DMR. Germ line DMRs acquire methylation during gametogenesis, and somatic DMRs acquire methylation after fertilization.[124] The *H19-Igf2* DMR is established in the germ line and is only methylated on the paternal allele.[130] Within the DMR, there are four binding sites for the insulator protein, CTCF.[145] CTCF can only bind the maternal DMR, which is unmethylated, but not to the methylated paternal allele. This inhibits maternal *Igf2* expression through blocking communication between the *Igf2* promoter and the shared *H19-Igf2* enhancer.[145] Mutation of the CTCF binding sites within the ICR results in biallelic expression of Igf2 and no expression of H19, revealing that CTCF binding is necessary for control of biallelic expression.[131,146]

Differential methylation within the ICR of the *H19-Igf2* locus is essential to maintain parent-of-origin expression. However, the contribution of histone modifications cannot be ignored. The histone code within this region has been mapped, and specific modifications are associated with specific alleles and transcriptional states. Within the maternal allele, the *H19* locus is enriched for modifications associated with active chromatin (i.e., H3K4me3), while the paternal allele is enriched with repressive marks (i.e., H3K9me3).[123] However, within the paternally expressed *Igf2* promoter, active chromatin marks pervade, while the same region on the maternal allele is enriched with repressive marks.[147–149] Histone modifications may help maintain the proper chromatin state, which helps to activate or repress the gene in an allele-specific manner.

Noncoding RNA Model of Imprinting Control

While DNA methylation is the most highly studied epigenetic regulatory epigenetic mechanism involved in imprinting, it is becoming apparent that ncRNAs are important for maintenance of monoallelic expression. At least one gene in imprinted clusters is a macro ncRNA.[136] These ncRNAs can either act as either short- or long-range silencers.[150] The short-range ncRNA transcripts partially overlap the one of the regulated genes, while with the long-range ncRNAs there is no overlap. An example of a functional ncRNA is that of the *Airn* gene, which helps to regulate the *Igf2r* imprinting cluster. In the placenta, *Igf2r* is expressed only from the maternal allele, as well as two other genes within the imprinting cluster, *Slc22a3* and *Slc22a2*.[136] *Airn* is an ncRNA within this imprinting cluster and is only expressed from the paternal allele. *Airn* is a short-range ncRNA as the transcript overlaps with the promoter of *Igf2r*. The necessity of *Airn* expression in the maintenance of monoallelic expression was shown in an elegant experiment where only a truncated form of *Airn* could be expressed from the paternal allele.[151] Without the full-length transcript the paternal alleles were expressed, and monoallelic control was lost at this imprinted locus. Expression of the truncated allele exhibited imprinted expression, and the methylation within the *Airn* promoter remained unchanged. While evidence shows that ncRNAs are able to repress genes in *cis*, the mechanism(s) through which they act is under investigation.

Although only about 1% of all genes are imprinted, loss of proper monoallelic expression of these genes is deleterious to the developing embryo. Epigenetic mechanisms are essential in marking and controlling which allele is expressed and which is repressed. While DNA methylation is essential for maintenance of the genomic imprints, the contributions of both histone modifications and ncRNAs are beginning to be explored.

IMPRINTING DISORDERS

More than 30 disorders whose etiology arises from an imprinting disorder have been characterized.[152] Because imprinting disorders involve alterations in proper epigenetic patterning, and these patterns are disrupted

during fetal development, the study of imprinting disorders truly is a study of the fetal epigenetic origins of disease. A disease that is considered to have an epigenetic etiology is unique from one with a strictly genetic cause. For imprinting disorders, it is possible for the individual to have one unaltered/unmutated copy of an implicated allele. However, due to genomic imprinting, the "healthy" gene cannot be expressed and this leads to a phenotype. The expression of this gene is prohibited by an epigenetic mechanism, namely, DNA methylation within the imprinting control region.

In this section we will discuss the most well-characterized imprinting disorders, including PWS, Angelman syndrome (AS), Silver–Russell syndrome (SRS, also called Russell–Silver syndrome), and transient neonatal diabetes mellitus (TNDM1). While Beckwith–Wiedemann syndrome (BWS) is also a highly characterized imprinting disorder, we will discuss the clinical characteristics, genetic and epigenetic changes in the next section on imprinting disorders and ART.

PWS and AS

PWS was first described in 1956, but its genetic origins weren't discovered for 30 years after its first characterization.[153] It is a rare neurodevelopmental disorder, occurring in approximately 1:25,000 live births.[154] PWS patients go through two distinct stages, phenotypically, during development. From birth to approximately 3 years of age, the child is characterized by hypotonia, feeding difficulties, lethargy, and failure to thrive.[153] A switch to an excessive interest in food, coupled with excessive eating, marks transition to the second stage. While the height of the PWS patient will fall behind others in the age range, the weight will far surpass, due to the excessive hyperphagia exhibited by these patients. By school age, the PWS child will show signs of mild intellectual disabilities and social difficulties.[153] Early diagnosis is essential to alleviate some of the more severe phenotypes such as short stature, which can be alleviated with growth hormone supplements. Other phenotypes the PWS child may exhibit include temper tantrums, mood and sleep disturbances, and repetitive and ritualistic behaviors.

AS was originally described in 1965 and occurs in 1:12,000 births.[155] AS patients develop normally up to 6 months of age, but development severely declines shortly thereafter. AS patients display gait ataxia, severe mental retardation, seizures, hyperactivity, sleep disorders, and limited speech.[132,152] They generally display a happy disposition and are characterized by excessive and inappropriate laughter.[54] AS patients also have a characteristic EEG pattern.[57] Autism is described in approximately 40% of Angelman patients,[133,134] perhaps due to the exhibited communication deficits.

The 15q11q13 Region

Deletions in the same chromosomal region (15q11q13) were identified by chromosomal banding to be associated with both AS and PWS.[132] This discovery seemed at odds with the drastically different phenotypes exhibited by patients with the two different syndromes. In 1989, Knoll et al. published that the four AS patients tested in their study carried a deletion in this region inherited from the mother,[135] establishing a parent-of-origin effect that could account for the two syndromes arising from deletions in the same chromosomal location. This conclusion is supported by the fact that maternal uniparental disomy of chromosome 15 (mUPD15), where the offspring carries two copies of chromosome 15 inherited from the mother and none from the father, is found in some PWS patients.[156] On the other hand, some AS patients have pUPD15.[137]

The parent-of-origin effect arises from the fact that the deleted region in the aforementioned cases is part of a large cluster of imprinted genes. While the contribution of these genes to the etiology of PWS is rather complicated, the cause of AS appears to be much more straightforward. Within this imprinted cluster lies an E3 ubiquitin ligase gene, *UBE3A*. *UBE3A* is biallelically expressed in all tissues except for in the brain, where the paternal copy is silenced.[155] The majority of AS cases have been traced to a large (6 MB) deletion within the 15q13q11 region that contains *UBE3A*. However, mutation of the maternal allele of *UBE3A* alone has been found in approximately 15% of AS cases.[138] pUPD15 accounts for approximately 7% of AS cases, and deletions that disrupt imprinting only account for 2–4% of all AS cases.[139,157]

The mechanism of imprinting within the neurons does not involve differential promoter methylation between the maternal and paternal alleles.[139] Instead, repression of the paternal allele is achieved through expression of the antisense RNA, *UBE3A-ATS*, only from the paternal allele and only in neurons.[158] *UBE3A-ATS* directly mediates silencing of the paternal *UBE3A* allele in *cis*.[159] In mice, it is possible to activate the paternal *UBE3A* gene. This can occur through repression of the paternal antisense RNA, through deletion of the imprinting control region.[160] Perhaps future work on inactivating *UBE3A-ATS* will provide a treatment for this complex disease.[159]

While the etiology of PWS is not as well defined as that of AS, current data suggest that specific genes within the 15q13q11 imprinted locus contribute to PWS etiology. Approximately 70% of all PWS patients have a deletion of this chromosomal region on the paternally inherited chromosome, while 25% patients have mUPD15.[132] Studies of patients with microdeletions within this region have helped to further establish the contribution of specific genes within the imprinted cluster involved in the

PWS phenotype. Within this region, *MKRN3*, *MAGEL12*, *NDN*, *C15orf2*, *SNURF-SNURPN*, and the *SNORD116* cluster are all paternally expressed and maternally repressed.[132] A key cluster of genes in PWS appears to be the SNURF-SNURPN locus, which also contains a cluster of small nucleolar RNA (snoRNA) genes.

The SNURF-SNURPN locus encodes one mRNA, which is translated as two independent genes (SNURF and SNRPN).[161] The locus contains 10 exons: exons 1–3 encode SNURF, and 4–10 encode SNRPN. Within the introns of SNRP-SNRPN lie seven snoRNAs whose molecular function is still unknown.[140] Paternal expression of the SNURF-SNRPN locus is maintained through differential methylation within the PWS-ICR, where the maternal allele is methylated and the paternal unmethylated. The promoter region and exon 1 of SNURF-SNURPN overlap with the PWS-ICR.[132] While the snoRNAs within this region do not show differential methylation, it is likely their paternal specific expression is regulated through expression of SNURF-SNURPN.

Many studies have implicated the SNURF-SNURPN genes in PWS. Balanced translocations of this locus, as well as inactivation of the snoRNAs encoded within the locus, have been described in PWS.[57] Microdeletions within the PWS-ICR have also been reported in PWS patients. PWS has also been described in patients who have epimutations within this region without a chromosomal deletion. The patients all have a paternal chromosome that carries the incorrect maternal imprint.[141]

Transient Neonatal Diabetes Mellitus 1

First distinguished from the classical disease in 1962, TNDM1 has an incidence of 1:400,000–500,000 and accounts for about 40–50% of diabetes cases diagnosed at birth.[142,143] Infants with TNDM1 are characterized by severe intrauterine growth restriction (IUGR), hypoglycemia, and low insulin.[142,162] Interestingly, the diabetes resolves itself, on average, at about 3 months of age, and normal height and weight is achieved by 2 years of age.[125] Diabetes returns in approximately 50% of these patients between the ages of 4–23.[163]

TNDM1 is caused by an aberrant expression of the *PLAGL1* and *HYMA1* genes located at 6q24. In fetal tissues, these genes are only expressed from the paternal locus.[164] Expression of these genes is controlled through differential expression of the TDM DMR, where the unexpressed maternal allele is methylated while the paternal is not.[126] While AS and PWS are caused by loss of expression of imprinted genes, TNDM1 is caused by any genetic or epigenetic mutation that leads to overexpression, or two functional copies, of these two genes. TNDM1 has been described in patients with pUPD6, as well as patients who have a duplication of the paternal 6q24 locus.[127,142] Furthermore, loss of methylation of the maternal DMR can lead to expression of the maternal genes and has been described in some patients.[128]

Silver–Russell Syndrome

Also referred to as Russell–Silver syndrome, SRS is a complex imprinting disorder whose genetic and epigenetic etiologies remain poorly understood. The clinical characteristics are varied, but mostly include both intrauterine and postnatal growth restriction, relative macrocephaly, failure to thrive, low BMI, a triangular face with a prominent forehead, and limb and body asymmetry.[165] Children who do not exhibit catch-up growth by two years of age may be recommended for growth hormone supplementation.[166]

While no specific gene locus has been definitively implicated in the etiology of SRS, aberrations within two chromosomes have been reported in SRS patients. Approximately 7–10% of SRS patients have mUPD7.[167] One candidate region within chromosome 7 appears to be 7p11.2-p13, as patients with SRS have duplications of this region.[168] One candidate gene within this region is *GRB10* (growth factor receptor-bound protein 10), which is imprinted and shows complex expression in most tissues.[166] In mice, loss of maternal *Grb10* leads to fetal overgrowth while overexpression leads to growth restriction.[169,170] However, no mutation in *GRB10* in humans has been found to be associated with SRS.[171]

The role of chromosome 11 was discovered when two patients with SRS were reported to have duplications of 11p15.[172] Within this region lie the imprinted genes *IGF2* and *H19*. Mutations, UPD, and aberrant methylation within this region had already been described for BWS. Therefore, studies of methylation status within this region in SRS patients were initiated. Indeed, approximately 38% of SRS cases revealed hypomethylation of ICR1.[166] While this could potentially repress *IGF2* expression, serum levels of SRS patients of *IGF2* are unaltered.[173]

Implications

The role of imprinting in human disease is one that has frustrated scientists for decades. How have we evolved such that we can have a completely healthy copy of a gene, but because of imprinting the gene cannot be expressed and a disease state is inevitable? While many imprinting disorders have a genetic as well as an epigenetic cause, there are patients who only exhibit epimutations associated with their disease. The challenges for the scientist are to determine how to overcome this parent-of-origin effect in order to reestablish expression of the healthy copy of the gene in the diseased individual. The role of *UBE3A* in AS is a great candidate to consider. The AS patient likely has the healthy paternal copy of *UBE3A*

being expressed in all tissues except for the neurons. How can the paternal allele be turned on in the brain in order to alleviate the symptoms? How can we protect proper imprinting to begin with? There are many questions to ask regarding imprinting and human disease, and we have only just begun to consider the answers.

IMPRINTING DISORDERS AND ART

In 2003, three studies emerged with the same conclusion: incidence of BWS is increased in children conceived using ART. The results of these studies stimulated discussion and debate and prompted hundreds of follow-up studies to determine if ART is associated with imprinting disorders and altered DNA methylation patterns. Questions emerged whether the increased incidence was due to the superovulation, the culturing techniques, or perhaps to problems inherent in subfertile couples. Here we summarize the clinical characteristics of BWS, the known causes, and the studies of BWS incidence and ART. We further discuss the studies of other imprinting disorders, such as AS and PWS in children conceived using ART.

Beckwith–Wiedemann Syndrome

BWS is the most common pediatric overgrowth syndrome, characterized by macrosomia, macroglossia, abdominal wall defects, anterior ear lobe creases, and visceromegaly.[174,175] BWS children have a clinically recognized increased risk for tumor development before the age of eight, especially Wilms tumor and hepatoblastoma, but also including neuroblastoma, rhabdomyosarcoma, and adrenocortical carcinoma.[175] The etiology of BWS was first mapped to chromosome 11p15 in 1983,[128] and has since been rigorously characterized concerning imprinted genes and parent-of-origin expression. The changes within this region associated with BWS can be genetic (uniparental disomy and mutations) or epigenetic (loss or gain of methylation at imprinting control regions).[174]

Within chromosome 11p15.5 are two distinct functional domains of imprinting. Within Domain 1 lies two imprinted genes, H19 and IGF2, whose expression is controlled by methylation of an imprinting center, IC1, located between the two genes.[176] IGF2 is a paternally expressed growth factor, while H19 is a maternally expressed noncoding RNA. Expression of these genes is controlled through methylation of IC1 on the paternal allele, while the maternal IC1 is unmethylated.[176] Domain 2 contains five imprinted genes: *KCNQ1*, *KCNQ1OT1*, *CDKN1C*, *SLC22A18*, and *PHLDA2*.[176] Expression of these genes in controlled by IC2, located within intron 10 of *KCNQ1OT1*.[176] Sporadic loss of methylation at IC2 or gain of methylation at IC1 has been reported in BWS patients with and without accompanying genetic changes.[55,177,178]

Incidence of BWS with ART

Three studies published in 2003 implicated the use of ART with an increased incidence of the imprinting disorder BWS.[76,77,179] Because BWS is such a rare disease (0.7–1.2% in the general population),[77,179] and only 1–3% of all births involve ART,[180] concluding an association between BWS and ART is extremely difficult. While these studies have been criticized for their small sample size and lack of proper controls, they have initiated a cascade of studies that seek to determine the increased incidence, if any, of alterations in genomic imprinting and imprinting disorders that may be more likely in children conceived using ART.

The study by Maher et al. focused on the incidence of ART in a cohort of 149 BWS children who had been referred to the BWS research group in Birmingham, UK.[77] Within this group, six children had been conceived by ART, including three from intracytoplasmic sperm injection (ICSI) and three from in vitro fertilization (IVF). This incidence is equal to 4%, which is higher than the incidence found in the general population (0.7–1.2%). Furthermore, two of these cases were assessed and found to have loss of methylation of the maternal allele at the KvDMR1 locus. Very similar results were found in a study published by Gicquel et al.[77] They too studied a cohort of 149 patients with BWS and similarly found that six of the 149 patients were born after ART. All six showed hypomethylation of KvDMR1. A third study published in the same year found that three out of 65 patients in the Washington University BWS registry were conceived using ART.[179] This indicates an incidence of BWS at 4.6% following ART, approximately a sixfold increase over the reported 0.76% in the general population.

While these studies are of interest, the size of the population in all three studies is perhaps too small to make a broad claim that indeed ART is associated with increased BWS incidence. Since these studies have been published, others have followed suit, analyzing larger cohorts of BWS patients and including more rigorous control groups. In 2004, Halliday et al. performed a case control study to determine BWS incidence with ART.[181] In their study design, they started with 37 BWS patients born in Victoria, Australia between 1983 and 2003, which was a prevalence of 1/35,580 of live births. As controls, four randomly selected live-born infants were chosen for each BWS patient, and were born within a month of the case for a total of 148 controls. They found that IVF was the method of conception for four of the BWS patients (10.8%) and only 1 (0.67%) of the controls, with an odds ratio (OR) of 17.8% (95% CI 1.8–432.9), $p = 0.006$. Although this study shows an almost 18-fold greater

chance of BWS through ART, the confidence interval is rather large.

While the foregoing studies begin to make a convincing argument, other studies, using larger cohorts, have shown that ART and BWS are not associated. In 2005, Lidegaard et al. published a study including all live births in Denmark between 1995 and 2001.[182] Within this cohort were 442,349 non-IVF and 6052 IVF children. They found no incidence of BWS in either cohort. Similar results were found in a Swedish study in 2005.[183] Their study included 16,280 children born after IVF in Sweden between 1982 and 2001. They did not find any incidence of BWS in this cohort.

The published studies describing an association, or lack of association, between ART and imprinting disorders are conflicting, to say the least. While many studies show as association, larger-powered studies do not reveal an increased risk for imprinting disorders with ART. Because these disorders are so devastating, and because the number of individuals born using ART is increasing, a more definitive answer on this issue is essential. Likely, a large international study, including data from many centers worldwide, would provide a more concrete answer. Furthermore, it could be determined, knowing the cause of infertility, if the cause of the imprinting disorder was due to the issues that underlie the infertility or from the ART procedures themselves.

Incidence of AS with ART

While many of the first reports of ART and imprinting disorders focused on BWS, other imprinting disorders have also been studied. AS prevalence has been reported to increase with ART. In the general population, AS frequency is approximately 1:12,000 births.[180] However, imprinting defects only account for approximately 4% of AS cases.[184] The first two reports of AS association with ART described three children born after ICSI who had AS.[185,186] Two Dutch studies also demonstrated that AS children were more likely in subfertile couples (TTP > 12 months) with a higher relative risk (6.3%) with ART than in the general population.[184,187] In one study, out of 16 children with AS born to subfertile couples, four of them had sporadic imprinting defects.[184] In a second study, out of 63 children with AS, four had been conceived using ART.[187]

Implications

ART has not been associated with an increased risk for PWS or SRS.[188] However, the current evidence for an association between ART and BWS or AS is concerning. While clinical follow-up studies on BWS and AS patients conceived after ART must continue to determine the etiology behind their disorder, research into whether the ART process plays a role in increasing the incidence of imprinting disorders is essential.

THE DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE: MATERNAL CONSTRAINTS

Developmental Origins of Health and Disease (DOHaD) is a hypothesis that postulates that adverse experiences in utero can increase the susceptibility of the developing fetus to the adult onset of metabolic disease (Figure 45.4).[189] In 1998 Barker et al. published that blood pressure as an adult was significantly related to birth weight.[2] Many studies followed that confirmed that the in utero experience could influence adult disease. In this section we highlight studies from individuals born during the Dutch famine during World War II, as well as animal models that have studied epigenetic changes in utero with maternal methyl donor supplementation, protein restriction, obesity, and tobacco smoke exposure. These changes are summarized in Table 45.3

Exposure to Famine: The Dutch Hunger Winter of 1944–1945

Studies of individuals born during the Dutch famine give us a unique understanding of the effects of in utero exposure to maternal calorie restriction and starvation on birth weight and adult metabolic diseases. The Dutch famine, or Hunger Winter, occurred during a defined time period from late 1944 to early 1945. During

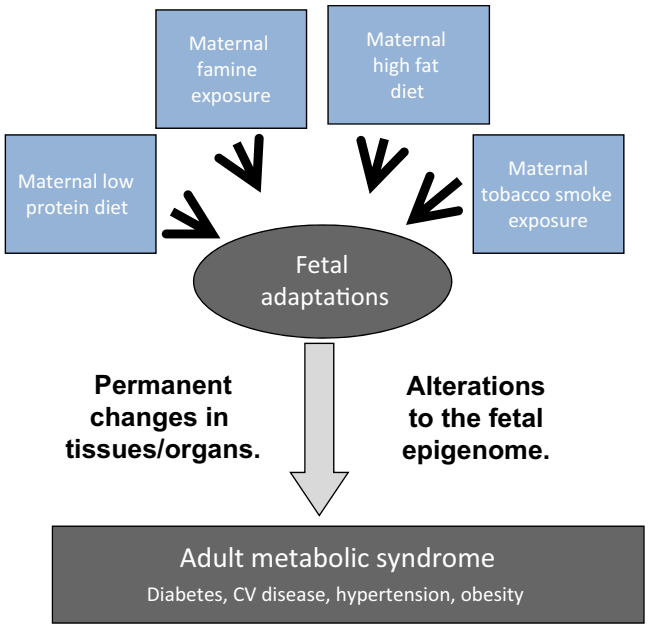

FIGURE 45.4 Maternal constraints: the developmental origins of adult health and disease (DOHaD). Many studies have shown that various maternal constraints are associated with the adult onset of metabolic disease such as exposure to in utero protein restriction, high fat diet, or famine. Epigenetic mechanisms may play a role in maintaining a memory of this in utero exposure until adulthood.

this time, areas of northern and western Holland were subject to starvation due to a Nazi-imposed embargo on food supplies. Because of this, the average citizen had only approximately 500 calories per day, and more than 18,000 people died from starvation.[212] Yet, throughout the famine, meticulous health care records were kept, including information on pregnant women and their newborns. Due to the meticulous records, the gestational period when the fetus was exposed to starvation (periconceptually, early gestation, late gestation) can be established. These individuals who were born during the Hunger Winter have been studied. Researchers have been able to determine phenotypic outcomes associated with in utero exposure to starvation. In many studies, unexposed siblings have served as the controls. In this section, we will summarize studies of the phenotypic outcomes of these individuals, as well as the epigenetic changes that have been recorded.

Besides the meticulous records kept during the famine, another unique aspect of this period is that once the famine ended, the population had an abundance of food. Individuals exposed to starvation in the womb were not born into a world where they would be exposed to starvation for a long period of time. Perhaps a disconnect between the fetal life (starvation) versus postnatal life (abundance) can account for the myriad published metabolic consequences these individuals experienced in adulthood. For example, in 1976 Ravelli et al. demonstrated that individuals exposed to the famine during the first half of gestation had an increased likelihood of developing obesity by age 19 ($p > 0.0005$).[213] Further studies on these individuals revealed a plethora of cardiovascular symptoms. Exposed individuals are more likely to have an atherogenic lipid profile, hypertension, disturbed coagulation, and a significant chance of developing coronary heart disease in adulthood.[190] The effects of the famine may even be transgenerational. In a study of women exposed to famine in the womb, their children showed increased neonatal adiposity compared with mothers who were not exposed in utero.[191]

The results of these studies beg the question: how is the memory of in utero exposure to starvation maintained for decades? Many researchers believe that epigenetics plays a critical role in establishing a molecular memory in the fetus that could be maintained into adulthood. To date, there are only two reports studying DNA methylation in individuals exposed in utero to the Dutch famine.[214,215] Both reports demonstrate a difference in peripheral blood DNA methylation levels at specific loci.

In 2008, Hejimans et al. used a quantitative mass spectroscopy analysis to determine if exposed individuals had differential methylation of the imprinted IGF2 promoter six decades after the famine.[215] Sixty individuals exposed in utero to the famine were enrolled, and their same sex siblings served as controls. Within the exposed individuals, four out of five CpG sites within the IGF2 DMR were hypomethylated compared with their siblings. A similar test of DNA methylation from 62 individuals who were exposed to the famine in only the last 10 weeks of gestation did not reveal a difference in methylation. Timing of exposure is likely important in setting these persistent epigenetic changes in utero. In a follow-up study of the same individuals, the methylation status of 15 genes involved in metabolism and cardiovascular disease was interrogated.[214] In this study, it was reported that methylation of *INSIGF* was decreased, while methylation of *IL10*, *LEP*, *ABCA1*, *GNASAS*, and *MEG3* were increased. These results show that the in utero environment is crucial in establishing DNA methylation of imprinted regions, and that disruptions during this period may actually be associated with life-long changes in the epigenome.

TABLE 45.3 Maternal Constraints and Epigenetic Changes

Maternal Constraint	Epigenetic Change	Model System	References
Famine exposure	Differential DNA methylation in peripheral blood	Human	188,189
Methyl donor enriched diet	Changes in DNA methylation at Agouti promoter	Mouse	190,191
Protein restriction	Fetal liver DNA hypomethylation	Rat	192
Protein restriction	Promoter specific changes in DNA methylation and histone modifications	Rat	193–198
High fat diet	Global and promoter specific hypomethylation in the brain	Mouse	199
High fat diet	Promoter specific DNA methylation changes in bone	Rat	200
High fat diet	Hypermethylation of cell cycle regulator in liver	Rat	201
High fat diet	Changes in levels and promoter specific H3K14ac	Monkey	202–205
Tobacco smoke	Promoter specific changes in DNA methylation and gene expression	Human	206,207
Tobacco smoke	Global DNA hypomethylation in cord blood from smokers	Human	208
Tobacco smoke	Changes in repeat DNA methylation in buccal cells and placenta	Human	209–211

Metastable Epialleles: Evidence for the Fetal Epigenetic Origins of Disease

Studies using the Agouti yellow mouse demonstrate how maternal diet influences epigenetic programming and can influence the adult onset of metabolic disease. These mice contain a "metastable epiallele" that coincides with a distinct phenotypic readout. An epiallele is an allele of a gene that differs from other alleles of the same gene by virtue of its methylation status. Metastable epialleles are epialleles whose DNA methylation pattern can be altered by the environment. The expression of a metastable epiallele is influenced by DNA methylation in the promoter region. Therefore, the epiallele can be differentially expressed in genetically identical individuals, simply based on the methylation status surrounding the gene.[216]

The *Agouti* gene (A^{vy}) in mice codes for a paracrine signaling molecule. In the absence of the gene product, the follicular melanocytes produce a brown pigment; in its presence, they produce a yellow pigment (reviewed in Ref. 217). Expression of agouti is regulated through methylation of nine CpG sites within its promoter; increased methylation silences expression, while decreased methylation promotes transcription. The readout of expression is simply the coat color of the mouse. In a scenario where the promoter is completely methylated, the agouti gene is silenced and the mouse is brown. When the promoter is unmethylated, the gene is expressed and the mouse is yellow. Partial promoter methylation yields a mottled brown/yellow coat color. Besides the change in coat color, an increase in body weight in mice expressing the *Agouti* gene has been observed.

Because agouti gene expression is related to the amount of promoter methylation, this model system is ideal for identifying factors that can alter the fetal epigenome during development, with varying maternal constraints. In 2003, Waterland and Jirtle found that supplementation of the maternal diet with potential methyl donors, including folic acid, B12, choline, and betaine led to changes in percent methylation of the agouti locus and changes in coat color in the offspring, compared with unsupplemented controls.[5] Similar results were reported from the Wolff lab.[7] Experiments where the maternal diet was supplemented with genestein, the major phytoestrogen found in soy, also revealed similar results.[218] Not only did the offspring exposed in utero to maternal genestein supplementation show increased methylation at the Agouti locus and decreased expression but the methylation persisted into adulthood and appeared to protect the offspring from obesity. Using the same mouse model, Waterland et al. performed an elegant experiment to show that methyl donors prevent the transgenerational amplification of obesity.[6] They found that the tendency for Agouti mice toward obesity was worsened as the gene passed through generations of obese mothers. However, supplementation of the maternal diet with methyl donors prevented obesity in the next generation.

Use of the agouti mouse model has provided strong evidence relating a fetal exposure to adult disease. Methylation of the agouti locus is essential for prevention of obesity in the adult mouse. Proper methylation is established in utero and can be altered with supplementation of the maternal diet with the methyl donors typically found in prenatal vitamins such as folic acid. Increased methylation at this locus is associated with the prevention of obesity in the adult mouse. These studies provide a firm foundation elucidating a role for epigenetics in adult disease and the importance of the in utero environment in establishing these epigenetic patterns.

Maternal Protein Restriction

Animal models have been crucial in elucidating the phenotypes of offspring exposed to a maternal low protein (MLP) diet. Much of the MLP diet research has been performed in rat models, using casein as the protein source. Traditionally, the low protein diets will have approximately half the protein found in the control diets. Decades of research have revealed that offspring exposed to MLP in utero will have permanent metabolic changes, including hypertension, decreased body weight, and altered glucose metabolism.[219,220] These rat models of MLP diet exposure have enabled scientists to characterize epigenetic changes associated with specific phenotypes of these offspring such as hypertension and altered glucose metabolism.

The hypertension phenotype in the offspring is a highly characterized outcome of MLP exposure. The offspring have hypertension throughout adulthood.[221] Bertram et al. showed that glucocorticoid receptor expression, an important regulator of blood pressure, is increased at least two-fold in the kidney in fetal, juvenile, and adult offspring.[192] Others have shown that the hypertension in the MLP-exposed offspring can be alleviated with supplementation of the maternal diet with glycine or folic acid.[193,222]

Epigenetic mechanisms may play a role in establishing these adverse phenotypes in utero. A study of global DNA methylation in the rat fetal liver revealed hypermethylation in MLP-exposed fetuses compared with control diet.[194] In rat pups, 6 days after weaning, the MLP-exposed animals had an increased expression of both glucocorticoid receptor (GR) and PPARα.[195] This increase in expression is associated with a decrease in promoter methylation in both genes. The GR promoter is also enriched for histone modifications of active transcription (H3K9ac, H4K9ac) and depleted for repressive marks (H3K9me2, H3K9me3).[196] The changes in

gene expression and DNA methylation are alleviated with supplementation of the MLP diet with folic acid.[195] Similar changes in expression and methylation are seen at the AT_{1b} angiotensin receptor gene.[197] Within the first week of life in rat pups, adrenal gland AT_{1b} expression is increased, while the proximal promoter is undermethylated after exposure to MLP diet.

Epigenetic mechanisms may similarly contribute to the altered glucose homeostasis phenotype also observed in these offspring. In this rat model system of MLP diet exposure, offspring undergo a gradual loss of glucose tolerance and develop symptoms of T2D by 17 months.[198,223] One mechanism behind this loss of tolerance could be an alteration in expression of hepatocyte nuclear factor 4-α (HNF4-α), a gene required for glucose homeostasis. MLP-exposed offspring, at both 3 and 15 months of age, show decreased HNF4-α expression in islet cells compared with controls.[224] This decreased expression is accompanied by an increase in promoter DNA methylation, a depletion of the activating histone modifications (H3ac and H3K4me1) and an enrichment of the repressive H3K9me2. Also, MLP-exposed offspring have an increased expression of CCAAT/enhancer binding protein β (C/EBPβ) in skeletal muscle.[225] C/EBPβ is an important transcription factor driving expression of insulin-responsive genes. The increase in expression is accompanied by an increase in both H3 and H4 acetylation within the promoter.[225]

While these aforementioned studies have demonstrated promoter-specific epigenetic changes with MLP-diet exposure, the most compelling evidence that epigenetics is involved in programming metabolism, and therefore adult disease, comes from studies showing that the effects of MLP exposure are transgenerational. Two separate groups have tested the F2 offspring using a rat model of maternal low protein diet exposure in utero, and both found similar results. In both experiments, the grandmother (F0) is given a low protein diet during gestation, such that the daughters (F1) were exposed in utero to MLP. After weaning, however, all F1 mothers consumed a control diet. In the next generation (F2), who were not exposed to MLP in utero, abnormal glucose metabolism is observed. Martin et al. demonstrated that the F2 generation from the F1-exposed MLP mothers had an increased insulin response at 30 and 120 min after a glucose challenge, compared to control F2 offspring.[226] Zambrano et al. found that the F2-female offspring of F1-MLP-exposed mothers showed evidence of insulin resistance.[227] A third group was able to show that fasting glucose and insulin levels were disrupted in the F3 generation, even though only their grandmothers were exposed in utero to an MLP diet.[228]

The rat model of maternal low protein diet exposure has offered compelling evidence that epigenetic alterations can be altered in fetal life in response to a suboptimal in utero environment. The modifications may be setting a memory of the in utero exposure that can last through adulthood. Because the altered glucose metabolism is seen in both the unexposed F2 and F3 generations, a likely epigenetic mechanism is transmitting this "exposure memory" throughout the germ line. It is unlikely that this transmission involves changes to the genetic code itself.

Maternal Obesity and Dietary Exposure

Although animal models of protein and calorie restriction have been studied for decades, in the era of the current obesity epidemic, animal models of maternal overnutrition have become of extreme importance. Over the past three decades the United States has seen a dramatic rise in the prevalence of obesity. Although it appears that this increase is leveling off, the most recent report from the CDC indicates that 68% of American adults are overweight or obese.[229,230] Obesity increases the risk for a number of health conditions, including metabolic syndrome and nonalcoholic fatty liver disease (NAFLD).[231]

While obesity is associated with adverse health outcomes for the individual, obesity during pregnancy bears unique maternal and fetal risks. The incidence of obesity among pregnant women is estimated between 18.5% and 38.3%.[199,232] These women are more susceptible to preeclampsia and gestational diabetes.[199,232] Newborns of obese and overweight women are more likely to be large for gestational age (LGA) and have a higher birth weight.[231] We are quickly finding many detrimental health consequences to the fetus exposed to a maternal high fat diet (HFD) and obesity in utero. In this section we will highlight studies using animal models of maternal HFD exposure that have shown epigenetic changes in the offspring bone, brain, and liver that correlate with specific morbidities associated with metabolic syndrome.

Is it maternal obesity or the maternal diet that bears primary influence on the offspring? Animal models have shown us that offspring of mothers who eat a high fat diet during gestation will have a preference for a high fat diet compared with unexposed offspring.[233,234] Vucetic et al. analyzed expression of genes in different regions of the brain that are involved in the consumption of palatable foods. They found that expression of the dopamine reuptake transporter, the μ opioid receptor, and preproenkephalin were all altered with HFD exposure in the adult male offspring exposed in utero to a maternal HFD.[200] They also found global and promoter-specific hypomethylation in the brains of these mice. This could serve as an epigenetic mechanism with the potential to reprogram a propensity to obesity in the offspring.

Another study of the deleterious effects of maternal HFD on offspring investigates an epigenetic predisposition to decreased bone mass and increased fracture

risk. Studies have shown that maternal HFD and obesity inhibit fetal skeletal development.[201,235] Chen et al. showed that fetuses of obese rats have inhibited skeletal formation at embryonic day e18.5 compared with controls.[236] The fetal cells involved in osteogenesis showed a decreased expression of HoxA10, a gene implicated both in fetal bone development and adult bone maintenance. Associated with this decreased expression, the authors found increased promoter methylation of the HoxA10 gene. They showed similar results in cell culture. Treating stromal-derived ST2 cells with free fatty acids showed an increased methylation of the Hox10A promoter compared with untreated cells. If the osteogenic cells respond to maternal lipids through methylation of the genes required for fetal skeletal formation, this could provide a mechanistic link between maternal HFD and inhibited skeletal formation.

Many studies have addressed the damage to the fetal liver with maternal high fat diet exposure. In rodents, it is well characterized that HFD exposure results in a high susceptibility to NAFLD in juvenile life and adulthood.[202] Dudley et al. studied the cell cycle in the postnatal rat liver of HFD-exposed offspring and found a significant cell cycle arrest in the G0/G1 phase at postnatal day 2 (P2).[203] In the HFD-exposed liver they found a decreased expression of the cell cycle inhibitor Cdkn1a, associated with promoter hypermethylation. However, the changes in cell cycle arrest, expression, and methylation observed in the P2 animals were not observed at P27.

Work in our laboratory has revealed many epigenetic changes in the nonhuman primate (NHP) fetal liver exposed to a maternal HFD. In this model, we are able to tease out epigenetic changes due to maternal obesity and those from a high fat diet. An NHP model closely mirrors human gestation. The Japanese macaques typically have a singleton, 6-month gestation, in contrast to the 21-day gestation in the mouse, where 8–12 pups per litter is common. In our NHP model, the dams are either on a control or a high fat diet throughout successive gestations. During this time the dams progress from overweight to obese. A subset of these obese dams is then switched to the control diet during breeding and throughout gestation. Because dams in this "diet reversal" cohort remain obese, we are able to determine the molecular effects of HFD versus maternal obesity.

Using this NHP model we have shown that the HFD-exposed fetus, at the beginning of the third trimester, has the pathology of NAFLD.[204] In an effort to determine if there are any epigenetic changes associated with NAFLD in the HFD-exposed fetal liver, we performed western blots on fetal liver lysates using antibodies specific for a myriad of histone modifications. We found that H3K14ac was uniquely enriched in the HFD-exposed fetal liver.[205] This increase in H3K14ac, while associated with HFD exposure, was not found in the diet reversal cohort.[237] In an effort to determine the mechanism behind the increase in K14ac observed in the HFD animals, we analyzed mRNA, protein, and activity levels of the known protein deacetylase SIRT1.[237] We found that all SIRT1 levels tested decreased with HFD exposure, which correlates with the increased K14ac levels. SIRT1 levels in the diet-reversal cohort were unchanged compared with controls.

Because we found an increase in H3K14ac in the fetal liver, we were interested to know which promoters were differentially enriched or depleted for this modification in HFD-exposed animals compared with controls. Using ChIP followed by differential display PCR we identified the *Npas2* gene, an important transcriptional regulator of circadian gene expression in the fetal liver.[205] Further investigation revealed that H3K14ac is enriched in the *Npas2* promoter only in HFD-exposed fetal liver and not in the diet-reversal cohort.[238] We found a similar promoter-specific enrichment of H3K14ac in the promoter of the thyroid hormone receptor beta gene, which drives transcription of thyroid hormone-dependent genes in the HFD-exposed fetal liver.[239] Our work on the NHP fetus shows that H3K14ac is a modification amenable in fetal life to maternal diet in the liver, and it is associated with specific promoters of important regulators of metabolism.

Similar to the results found in the offspring of protein-restricted mothers, some of the phenotypes of in utero HFD exposure can be transmitted transgenerationally. Interestingly, the effects of exposure can be transmitted by both the male and female offspring, as observed in a mouse model system.[240] Transmission of these phenotypes through the paternal germ line suggests an epigenetic mechanism. Dunn et al. found that the offspring of mothers and fathers who had only been exposed to HFD in utero had an increase in body length. These offspring also showed a reduction in sensitivity to insulin 30 min after an insulin tolerance test. Another study showed that obesity occurred earlier and was more severe in the third generation if both the mothers and grandmothers were raised on a HFD.[241] An increased propensity for obesity compared with offspring of control diet animals may suggest epigenetic reprogramming that occurred in utero with HFD exposure.

Maternal Tobacco Smoke Exposure

The epigenetic changes in the fetus associated with maternal tobacco smoke exposure (MTSE) have also recently been investigated. Despite decades of public health warnings, as many as 20% of pregnant women continue to smoke throughout gestation.[242] Since 1957 it has been repeatedly shown that smoking during pregnancy is associated with low birth weight babies and prematurity.[243,244] However, it has been noted that not all infants exposed to tobacco smoke in utero are born small

for gestational age (SGA). The mechanisms behind this differential susceptibility to MTSE are currently being explored. Specific genetic polymorphisms in genes that metabolize the xenobiotic compounds in tobacco smoke have been implicated in IUGR with MTSE.[245–247] Therefore, any epigenetic modification that alters expression of one or more of these genes could similarly contribute to IUGR susceptibility.[206,207] Here we highlight studies of epigenetic alterations associated with MTSE in placenta, cord blood, buccal cells, and peripheral blood.

In 2003, Antilla et al. published that expression of *CYP1A1*, the enzyme that metabolizes the polycyclic aromatic hydrocarbons in tobacco smoke, is highly upregulated in the lungs of smokers.[208] Within the *CYP1A1* promoter lies an essential element crucial for transcription called a xenobiotic response element (XRE). They found in the lungs of smokers that the XRE was hypomethylated compared with nonsmoking controls. We hypothesized that *CYP1A1* expression and promoter methylation would be similarly altered in the placenta from mothers who smoke compared with nonsmoking controls. Indeed, we found that *CYP1A1* expression is upregulated more than four-fold, and the XRE was hypomethylated by 10% compared to the nonsmokers.[209] Furthermore, methylation status only within the XRE correlated with *CYP1A1* expression. Methylation elsewhere within the 1.5 kb proximal promoter did not correlate with expression.

To see if other such genes showed a correlation between expression and methylation in smokers, we tested the DNA methylation and gene expression on a genome-wide scale from 18 smokers and 18 nonsmoker controls.[210] Methylation changed significantly at 1024 CpGs. We found that 438 genes had a significant correlation between methylation and gene expression in placentas from smoking mothers. These genes are enriched for members of the oxidative phosphorylation, mitochondrial dysfunction hypoxia response pathways. All of these mechanisms could potentially provide a mechanistic link between epigenetic changes and IUGR.

To date only one study has been published interrogating epigenetic changes observed in cord blood from smoking mothers. In an effort to determine if DNA methylation changes with MTSE, Guerrero-Preston et al. utilized an ELISA assay that measures global DNA methylation levels.[211] They correlated this data with measurements of cord blood cotinine levels, which is a reliable biomarker of maternal smoking. In this report they found that DNA methylation was highest in the cord blood from nonsmoking mothers and lowest in the heavy smokers. In other words, there was a negative correlation between DNA methylation and cord blood cotinine levels.

Only a few studies have looked at epigenetic changes in children or adults exposed in utero to MTSE. In two studies using buccal cells from kindergarten and first grade students, it was found that DNA methylation of the AluYb8 repeat is decreased, while methylation within the site-specific AXL gene is slightly increased.[248,249] This data corroborates a study in human placentas that showed that AluYb8 repeat DNA methylation is similarly lower in smokers than in nonsmokers.[250] Sat2 repeats show differential methylation in adult woman in peripheral blood granulocytes between those who were and were not exposed in utero to MTSE.[251]

THE DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE: PATERNAL CONSTRAINTS

For decades, research has focused on phenotypes in the offspring arising from various in utero, constraints such as maternal calorie restriction, low protein diet, or a high fat diet. More recently, however, a few publications have demonstrated that the father can also pass through the paternal germ line the "memory" of the exposure he experienced in utero. In other words, a male subjected to an adverse in utero environment can transmit that in utero experience to his offspring. Although such studies are not as plentiful, these recent studies are provocative and provide compelling evidence that epigenetic changes can be inherited through the germ line. In this section we will discuss Skinner's work on rats showing transmission of a paternal in utero exposure for four generations. We also highlight two separate studies showing paternal transmission of in utero exposure to protein restriction as well as postweaning exposure to an HFD.

Seminal work from Michael Skinner's lab used a rat model of endocrine disruption exposure in pregnancy to show that male offspring had a reduced fertility and that this phenotype was perpetuated for four generations.[252] In their previous experiments they had tested the effects of two known endocrine disruptors on pregnant rats and their offspring. They utilized methoxychlor, a pesticide, and vinclozolin, a fungicide used in the wine industry, which both have antiandrogenic activity. They found that a daily exposure of either chemical from E8–E15 resulted in the male offspring showing increased sperm apoptosis and a decreased sperm count.[253,254] However, daily exposure from E15–E20 did not show the same effect. They wanted to test if the F1 offspring who were exposed in utero to either methoxychlor or vinclozolin could transmit the phenotype of reduced sperm count and motility and increased sperm cell apoptosis.

In their experiment they crossed male progeny from an F0 treated mother with a female from an untreated mother. The male offspring of this cross showed a similar increase in spermatogenic sperm apoptosis, decreased sperm count, and increased motility.[252] A similar cross using female progeny from an F0 treated mother and a

male from an untreated mother did not have the same results. Continued crosses of the male offspring showed that this phenotype was transmitted through the F4 generation. These experiments suggest that epigenetic mechanisms were involved in transmitting the phenotype through the paternal germ line. Because 90% of all males in the F1–4 generations exhibited the phenotype, the frequency is too high to be explained by genetic mutation. Measurement of DNA methylation in epididymal sperm and testis did reveal slight changes in DNA methylation in the male progeny of F0 treated mothers.[252] The researchers concluded that the phenotype was only carried through the paternal lineage.

While we have highlighted many studies of maternal high fat diet exposure, one study has shown that the effects of postweaning paternal chronic consumption of a high fat diet are transmissible to female offspring.[255] Ng et al. showed that male rats chronically consuming a high fat diet showed increased body weight and adiposity and reduced glucose tolerance. The female offspring of these HFD-fed fathers also showed impaired glucose tolerance and insulin secretion but did not have an increased body weight. Transcriptional analysis of the β-cells in these female offspring revealed a significant change in 642 genes. The authors also tested promoter methylation of the gene that showed the highest fold-change in their analysis, *Il13ra2*. They found the increased expression was accompanied by promoter hypomethylation.

A final study that highlights transmission through the paternal germ line uses a mouse model of maternal low protein diet exposure.[256] In this model, male mice exposed in utero to the MLP diet are crossed with unexposed females. The livers of the offspring from this cross were analyzed for changes in gene expression at 3 weeks of age. They found that 1595 genes showed differential expression compared with offspring of unexposed fathers. Analysis of this gene set revealed an increase in expression of genes involved in lipid and cholesterol biosynthesis. Physiological analysis of the offspring livers revealed a two-fold decrease in cholesterol and cholesterol esters in these offspring. Because these changes in gene expression were transmitted through the father, the authors proposed an epigenetic mechanism is likely at play. They used high-throughput sequencing to look at miRNA levels in the offspring liver. While they found modest changes in a few miRNAs, they did not find expression changes in their expected targets. Analysis of approximately 1% of the mouse genome for changes in DNA methylation revealed a slight (20%) but significant change in methylation.

Transmission of phenotypes of environmental exposures by the father is an interesting and provocative discovery. In utero exposure to a low protein diet, or chronic consumption of an HFD, will not alter the underlying DNA sequence of the sperm genome. However, a distinct phenotype can be transmitted through the sperm. How epigenetic markings in the sperm can program the offspring is an important question in the field of the fetal epigenetic origins of disease and is likely a long way from being answered. As we have discussed previously in this chapter, the sperm chromatin is uniquely packaged and compacted, and very few histones remain within the paternal genome. Could the remaining paternal histones transmit such phenotypes to the offspring? Or are there changes in DNA methylation that occur in response to an environmental insult that are retained and somehow protected against the active demethylation event after fertilization? Currently, the study of transmission of environmentally derived phenotypes through the paternal genome is still very young. Many more studies will be required to observe the extent of the paternal transmission before a mechanism can be elucidated.

AREAS OF EMERGING INTEREST

The Microbiome: Important Player in the Fetal Epigenetic Origins of Disease?

The "human microbiome" is comprised of all the microorganisms present in the human body that exist in symbiotic (of benefit to the host) or nonsymbiotic (commensal or pathogenic to the host) relation in a complex ecosystem.[257–259] For several centuries, microbes were viewed primarily as pathogens in a negative context to causation of human diseases. However, recent advances in sequencing technology have enabled scientists to catalog all the organisms (culturable and nonculturable) present in the human body, and we have expanded this work specifically among pregnant women.[260–265] Suffice it to say, we now appreciate that the microbial genome exceeds the human genome by at least 150-fold, and this community genome (or "metagenome") encodes for an antigenically and metabolically vibrant community.

While these impressive efforts to date have cataloged the human microbiome and its carriage patterns, we are only beginning to understand how the microbiome is established and what influences its composition. A few studies have shown that changes in the maternal microbiome may have protective effects on the infant. For example, Blumer et al. exposed pregnant mice to lipopolysaccharide, a component of the gram negative cell wall.[266] The neonates showed enhanced neonatal IFN-γ, but not IL-4 and IL-2 production, which may have implications for lessening the severity of allergies in the offspring. One study in humans has indicated that prenatal use of antibiotics may be associated with allergies in the offspring.[267] It has even been shown using probiotics that colonizing the mother with *Lactobacillus rhamnosus*

strain GG in late pregnancy can similarly colonize the neonatal gut.[268] During pregnancy, the maternal gut microbiome is related to body weight, weight gain, and metabolic biomarkers, and could therefore influence fetal health.[269] Such studies indicate that the maternal microbiome is having an effect both on offspring health and microbiome. In other words, we are now beginning to appreciate how human behavior and exposures shape the maternal microbiome, which in turn is capable of shaping the microbiome in the next generation.

While studies of the maternal microbiome are gaining interest, studies of how the microbiome associates with epigenetic changes are rare. Schaible et al. investigated whether maternal methyl donor (MD) supplementation changes the offspring susceptibility to colitis in mice.[270] They found that indeed, not only did these offspring have an increased susceptibility to colitis, they also had an altered gut mucosal transcriptome as well as changes in DNA methylation. However, at postnatal days 30 and 90 they did not show changes in the gut bacteriome. The role of MD supplementation on changes in DNA methylation, and subsequent colitis susceptibility, will be important to elucidate in future studies. In another study, Kellermayer et al. interrogated the gut microbiomes of WT mice and mice lacking the Toll-like receptor 2 (*Tlr2*$^{-/-}$) gene, a known modulator of gut tissue integrity.[271] Not only did species bacterial abundance differ between the two groups but they also had differential gut mucosal transcription and DNA methylation. Whether the epigenetic changes are associated with the change in gut flora or the genetic mutation of the mouse remains to be determined.

Obviously if the microbiome influences the epigenome or vice versa, there are many factors at play. Both have an important role in regulating fetal health and perhaps increasing the offspring's susceptibility to adult disease. We fully anticipate that in the very near future our understanding of the role of the microbiome–epigenome interactions in shaping reproductive health will have already expanded significantly. We further predict that this will be one of the most influential arenas for high-impact research in the history of developmental biology and reproductive outcomes.

Micronutrient Supplementation

We have highlighted studies of supplementing the maternal diet with methyl donors in animal models. However, many scientists are interested in whether similar changes occur in humans, especially in the context of prenatal vitamins, which typically provide many of the nutrients involved in one-carbon metabolism, including folic acid. Because the few available studies on humans hint that offspring DNA methylation changes are associated with micronutrient supplementation of the mother, many studies are warranted to tease out which doses and timing of supplementation are best.

One study that measured the two DMRs within the *IGF2* gene in umbilical blood found differential methylation between newborns from mothers who started taking prenatal vitamins before pregnancy compared with those who started only after conception.[272] Further studies have shown that methylation of LINE-1 repeat elements in cord blood DNA correlates with homocysteine levels and birth centile.[273,274] A study of micronutrient supplementation in Gambian women, which provided 14 different vitamins and minerals, revealed differential cord blood methylation compared with newborns from women taking a placebo.[275] The changes occurred in genes associated with immune defense, and could reveal an important mechanism through which epigenetics can predispose offspring to either health or disease.

CONCLUSION

There are major questions yet to be resolved regarding the epigenetic contributions to the developmental origins of health and disease. Numerous studies have chronicled changes that occur in the offspring with various in utero constraints. However, longitudinal studies as to how long the modifications last over the life span of the individual are lacking. If epigenetic modifications serve as a molecular memory of an in utero experience, it is important to know how long they persist throughout development and aging of the individual. Secondly, the question of how these epigenetic changes can be correlated with the phenotype observed in adulthood is unknown.

In humans, adult phenotypes likely have many converging etiologies, and determining the contribution of the in utero experience is difficult. However, animal models have their limitations as well. Being able to translate our observations from the mouse, rat, and monkey models to humans is also difficult. We are currently unable to determine if epigenetic changes that occur in utero are a consequence or cause of later-in-life phenotypes.

In the future, studies that follow epigenetic changes in individuals over the course of their lifetime, spanning several decades, will be essential to determine how persistent these epigenetic changes are. For example, DNA methylation changes have been reported in cord blood from newborns, buccal cells from children, and blood cells from adults after in utero exposure to maternal tobacco smoke. Whether these modifications are stable or change over the life span of the individual is unknown. However, this knowledge would aid in understanding the persistence of the changes in DNA methylation, and would further our understanding of such changes as a molecular memory of the in utero experience.

In the decades since Waddington initially coined the now common term epigenetic, our advances in reproductive biology and development have led us to discover the molecular epigenomic mechanisms that drive much of human adaptation, evolution, and developmental plasticity. The field has advanced from one of primarily phenomenology and observational descriptions to its current state of broad application to human health and disease. We anticipate that the "current" state of the science as described herein will similarly likely be historically relegated in a matter of a few short years as the fields of epigenomics, metagenomics, and comparative genomics rapidly merge to reveal the incredible grandiose beauty inherent to evolution—as observed one generation at a time. It will now be incumbent upon all of us who serve as reproductive biologists and clinician scientists to join in dynamic conversation with our social science, nutritional science, and behavioral science colleagues. Together we can expand this dialog to our public officials, community health workers, and policy makers to work ardently within our communities to push forward positive changes in diet and lifestyle modification across all strata and across the globe to be agents of change for better health and less disease burden in the years and generations to come. We speculate that we have only begun to see a fraction of the developmental landscape that is affected by the rapidly changing world in which we live, and have yet to fully appreciate the impact of environmental exposures on our metagenome and epigenome. We have the utmost confidence that with such dialog we will see a greater responsibility for the health of our population be assumed by those with the likely greatest ability to enable change.

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CHAPTER

46

Lactation and its Hormonal Control

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INTRODUCTION

Breastfeeding offers significant advantages to both infants and their mothers as elegantly reviewed in a recent article by Mohammed and Haymond.[1] However, breast milk production is often compromised in those mothers whose children might benefit most by breastfeeding according to the recommendations of the American Academy for Pediatrics, the Centers for Disease Control and the World Health Organization. These organizations all recommend exclusive breastfeeding for the first six months of infant life with supplementation with appropriate complementary foods up to one year of age.[2] Breast milk has significant benefits for at-risk populations such as preterm infants and those born to mothers with diabetes, metabolic syndrome, and obesity,[2,3] as well as for mothers with gestational diabetes mellitus.[4] Breastfeeding is critical in developing nations where clean water and resources to purchase formula are in short supply. We are beginning to understand that nonnutritive components of breast milk offer significant benefits to both the infant and the mothers that exceed the nutritional components present in this complex fluid.

The nutritional status of the mother prior to conception and during pregnancy has profound effects upon mammary gland development and function, as well as neonatal development. While infants born to nutritionally deprived mothers have long been a focus of public health policy, it is clear that at-risk infants also include preterm infants, and those born to mothers with diabetes, metabolic syndrome, and obesity. Given the importance of lactation to human health, it is increasingly important that we understand mammary gland development and the physiology of lactation in a manner that can guide public health policy. In this chapter we will examine lactation physiology from a variety of perspectives, with an initial focus on the understanding of molecular mechanisms gained by in-depth analysis of experimental models and protein interactions. We will proceed to metabolic interactions that lead to the increased flux of substrate to the mammary gland for milk synthesis and end with a summary of milk ejection mechanisms. We will also describe recent approaches to lactation research in humans, emphasizing some of the unique components of human milk and some new technologies that are beginning to provide molecular insight into human lactation.

Much of the early physiological research in lactation was carried out in dairy animals. Sometime in the 1950s, Jim Linzell, working at the Agricultural Research Council of the Institute of Animal Physiology at Babraham, Cambridge, UK, began to examine the blood flow in the mammary gland of the goat. Along with a young researcher, Malcolm Peaker, he soon began to combine blood flow measurements with new techniques that allowed metabolic reactions to be traced with radioactive isotopes in the animal. These experiments, summarized in *Physiological Reviews* in 1971,[5] provided insights into the mechanisms of milk secretion that had been undreamed of even a decade earlier. Around the same time, a world away in Berkeley, California, another researcher, Dorothy Pitelka, was applying her skills with the new miracle of microscopy, the electron microscope, to an examination of the morphology of the mammary gland at the minutest level.[6] Pitelka used her skills with transmission and freeze fracture electron microscopy to publish a series of papers that essentially defined cell–cell contacts in the mammary gland. She then went on to establish tissue culture models that would allow study of the regulation of these contacts and other aspects of the molecular control of the synthesis of milk proteins even to the present.[7] One of the exciting moments in the field was when Linzell and Pitelka both presented their work at a Gordon Conference in 1973, where each discovered that the other was working on complementary aspects of

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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tight junction regulation in the mammary gland, providing a framework for the work on tight junction regulation in the mammary gland that continues today. Many other researchers, too numerous to mention in this introduction, have contributed to our current understanding of the physiology of lactation, and some of their work will be described later in this chapter.

We are once again at a strategic junction in our understanding of the biology of milk secretion. During the decades leading up to the 1970s, biochemists identified and characterized the numerous enzymes that underlie the ability of the mammary gland to make milk. The application of molecular techniques during the 1980s allowed the cloning of genes for proteins known to be present in milk or to participate in synthesis and secretion of milk as well as identification of the promoter elements that regulate expression of these genes. The 1990s brought the identification of signal transduction pathways that control expression of specific genes and the differentiation of the mammary gland. The use of genetically modified mice confirmed the importance of these signaling molecules and pathways in regulating developmental processes during puberty, pregnancy, lactation, and involution, providing a better understanding of mammary gland development at the molecular level. In the last decade gene expression profiling, comparative genomics, and analysis of single nucleotide polymorphisms have produced a more complex picture of lactation that includes an appreciation of cross species differences and variation. The use of metabolomics is allowing a new appreciation of mammary physiology in a whole body context. Finally, the ability to localize specific molecules within the mammary cell using immunohistochemistry is greatly expanding our knowledge of the mechanisms of milk secretion. Our goal in this chapter is to make available a scaffold composed of past and current knowledge of the mammary gland providing a foundation for future advances that utilize biochemical, molecular, cell biological, nutritional, and genetic approaches in an integrated manner and advance our understanding of lactation and its importance to neonatal development.

DEVELOPMENTAL ASPECTS OF MILK SECRETION

Methods for Studying Mammary Gland Development and Function

Histological Methods

Examination of the primary structure of the mammary gland has long been the first approach to analysis of developmental alterations that affect glandular function. In fact, characterization of glandular function without examining the morphology of the gland for changes that underlie changes of function is likely to lead in erroneous directions. The first standard approach is preparation of whole mounts of the mammary gland allowing determination of whether ductal elongation, alveolar budding, and alveolar expansion have taken place (Figure 46.1). To do this, the whole mammary gland is excised, fixed, stained with either carmine alum or hematoxylin, and clarified to remove excess stain in such a way that the ductal tree can be appreciated (for review of methods see Ref. 10). Under low power it is possible to discern many aspects of mammary gland development particularly during puberty and early pregnancy before the florid development of late pregnancy and lactation make it difficult to discern individual ducts and alveolar units.

Conventional examination of thin tissue sections that have been stained with hematoxylin and eosin remains highly informative as the fine details of terminal end buds (TEB) and secretory alveoli are readily evident. While lipid droplets are readily identifiable in sections stained in this manner, greater detail is revealed when a combination of stains is used to identify various structures. For example, the use of anti-adipophilin antibody to outline cytoplasmic lipid droplets (CLDs), DAPI (4′, 6-siamidino-2-phenylindole) to stain nuclei, and fluorescent tagged-wheat germ agglutinin that binds to the luminal surface of secretory alveoli has allowed investigators to demonstrate that the size and position of CLDs changes during the transition from pregnancy to lactation[11] (see following). The combined use of antibodies to specific proteins with cytological markers for the nucleus, mitochondria, or other organelles will continue to provide in-depth understanding of cellular and signaling events in mammary gland development and lactation.

Transplantation Methods

The fact that the majority of mammary gland development occurs postnatally offers the unique opportunity to use transplantation to study the development of mammary epithelia containing targeted mutations.[12,13] In this method the mammary fat pad of a three-week-old prepubertal mouse is cleared by removal of the region of the gland proximal to the nipple, leaving a fat pad devoid of precursor cells capable of developing into the ductal tree (Figure 46.2). In the majority of studies a section of mammary gland from another mouse is placed into the cleared fat pad to develop in this new environment. Development is assessed at varying times following transplantation by whole mount analysis using contralateral glands on the same mouse as a control. Numerous variations on this basic technique have been developed over the years, including transplantation of tissue into non-cleared glands, transplantation of partially purified mammary "stem cells", and transplantation of "marked" cells expressing different fluorescent proteins or other

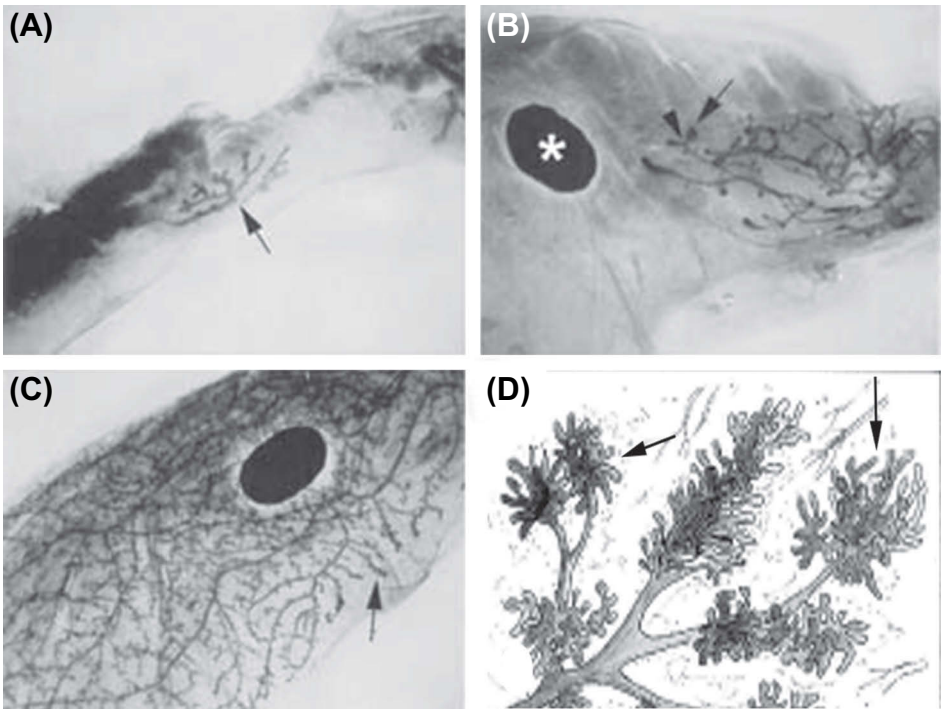

FIGURE 46.1 Whole mounts of mammary glands from virgin mice and humans. (A)–(C) The fourth inguinal mammary glands were dissected from female mice at the indicated stages and stained with hematoxylin. (A) 3-week virgin. The arrow indicates the nipple region and the primary duct of the epithelial structure. 10× magnification. (B) 5-week virgin. Asterisk marks the lymph node, commonly used as a marker in whole-mount analysis. Ductal growth is indicated by the TEBs (arrow) and branch points (arrowhead). 45× magnification. (C) 10-week virgin. Alveolar buds are forming along the ducts (arrow). 45× magnification. (D) Human, drawing of a subgross preparation of a mammary gland from a 22-year-old nulliparous female. Arrows point to terminal duct lobular units (TDLUs). *Source: (A)–(C) From Ref. 8. (D) From Ref. 9.*

detectable markers such as β-galactosidase. Transplantation studies have been critical in the identification of cell autonomous features, and in determining the role of the surrounding stromal environment in mammary gland development.

Methods to Assess Lactation Competency

Since the primary purpose of lactation is to support the growth of neonates, neonatal growth remains the best indication of lactation competency in most experimental species, although milk production can easily be assessed in dairy animals. Comments here are directed toward rodent experiments. Although it is simple to measure the weight of the litter and track its increase on a daily basis, several considerations should be given to this process so that meaningful data are obtained. First, it is important to standardize all litters to the same number of offspring so that each dam experiences an equivalent demand for milk; in the mouse, many investigators have standardized litters to eight pups, although for some lower producing strains six pups are more relevant.[15] Second, it is important to utilize pups without a developmental defect, often achieved by cross-fostering litters from a normal dam. When pups are well fed, the milk-filled stomach is readily apparent. Pups are either weighed individually or as a group, and the weights plotted over time. Numerous genetically modified mice display lactation defects when pup growth is used to access lactation competency; these include transgenic dams overexpressing constitutively activated AKT1,[16] *Akt1* null mice,[17] thyroid hormone responsive protein (THRSP or SPOT14) null mice,[18] and mice lacking tissue-specific expression of SREBP cleavage-activating protein (SCAP),[19] among many others.

Another approach is the "weigh-suckle-weigh" method in which pups are withdrawn from the dam for a defined period of time, such as 4 h, the pups weighed immediately before being returned to the dam, and then weighed again after 1–2 h of suckling. This method allows analysis over a defined period of time, sometimes in response to a specific treatment or intervention (for example, treatment with bromocriptine to disrupt prolactin (PRL) secretion[20]). Yet another approach is to weigh the excised mammary glands; however, results of this approach are likely to be influenced by the amount of time since suckling has occurred.[15]

In addition to analysis of pup growth, there is much to be learned by analysis of milk or milk curd itself.[15] Milk can be obtained from a lactating mouse with use of a suction device and oxytocin (OT).[21] The amount of

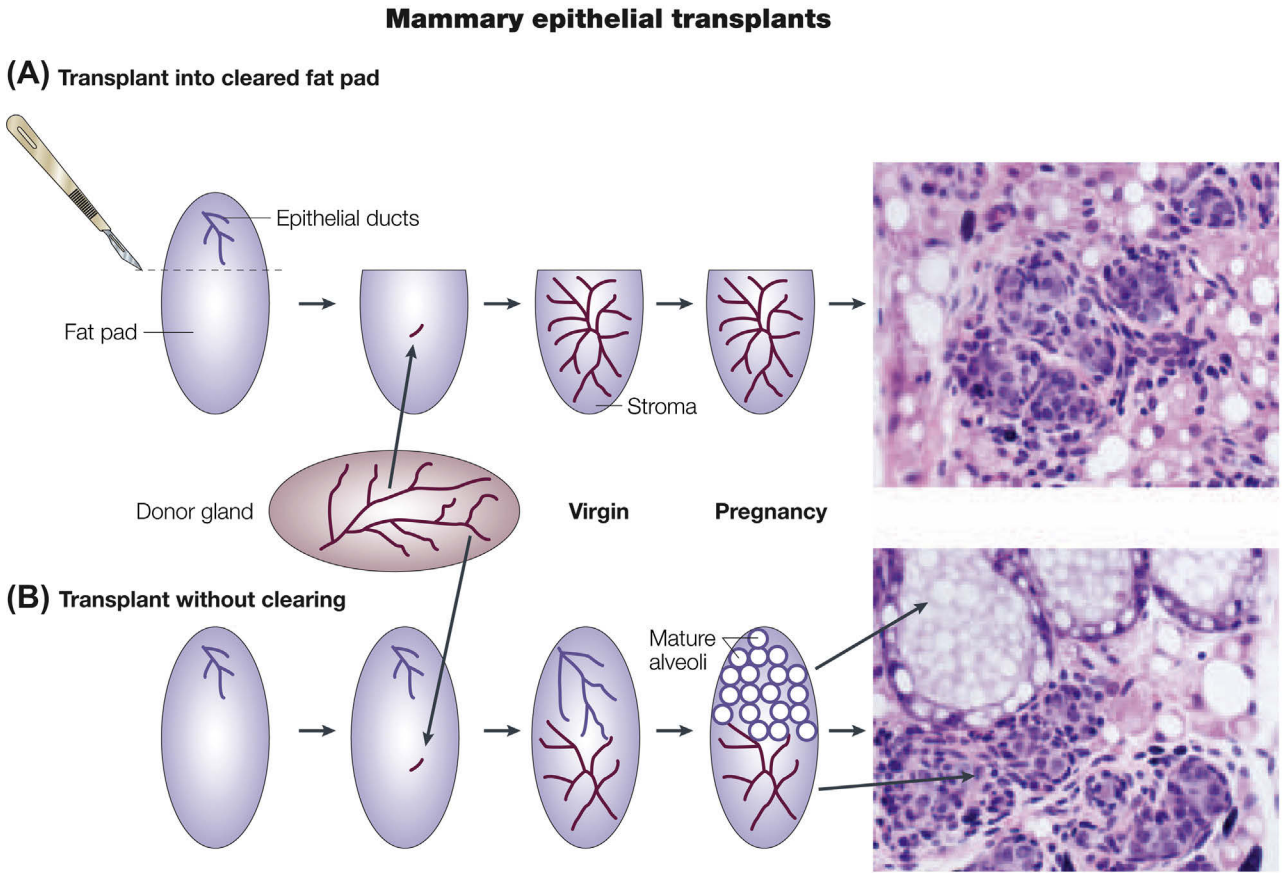

FIGURE 46.2 Use of mammary gland transplantation to assess developmental potential. This figure is reproduced in color in the color plate section. (A) The area containing the ductal anlage (purple) in a 3-week-old mouse is surgically removed to generate a "cleared fat pad" into which donor tissue (dark pink) is transplanted. The development of the transplanted tissue is monitored at intervals posttransplant by either whole mount or standard histological analysis. (B) An alternative approach involves direct transplantation into a non-cleared mammary gland. Transplanted cells are frequently marked with either immune-fluorescent markers, genetic markers, or infected with viruses expressing detectable markers. *Source: Used by permission from Macmillan Publishing Company; Ref. 14.*

protein present in the milk and the protein species present can be readily determined by gel electrophoresis. The milk lipid content can be determined on a volume basis using whole milk. Analysis of the milk clot from the stomach of pups after nursing is also possible, although effects of digestive enzymes make precise findings less reliable, or on dry weight basis using milk clot analysis. Furthermore, gas chromatography–mass spectroscopy can be used to determine the precise fatty acids present in the milk and their relative or exact concentrations.[19,22] New technologies are allowing precise determination of increasingly important components of milk, such as oligosaccharides, growth factors, cytokines, and glycosylated proteins.[23,24]

Methods for Studying Human Lactation

Studies of the physiology of lactation in humans have the advantage of direct clinical relevance. Further, milk is available in quantities that allow precise quantitation of nutrients and other molecules. They are made more difficult by the complex methods needed to accurately quantitate milk volume transfer to the infant and the near impossibility of obtaining samples of mammary tissue in a systematic fashion at various stages of mammary development. Our understanding of the histology of the human breast has come mostly from autopsy studies[25] and from samples of normal tissue taken in association with breast cancer diagnostic procedures. However, breast milk is available at all stages of lactation and offers considerable insight into both the complex composition of this fluid and its changes with time postpartum. Recently, insight into molecular mechanisms has been obtained from analysis of milk fat globule (MFG) membranes; these contain significant amounts of mammary cell cytoplasm, allowing assessment of mRNA expression.[26] In addition, the process of secretory activation takes place after birth rather than prepartum as in most species, allowing the molecular underpinnings of this process to be studied by careful analysis of milk components.

Breast milk production can be assessed in three ways: (1) In mothers who are pumping their breasts to obtain

milk for a preterm or ill infant, the volume of milk pumped is easy to measure, most accurately by weighing the milk container before and after pumping. This technique allowed researchers to determine that retained placental fragments can inhibit secretory activation in women, most likely by their secretion of P4.[27] (2) Willing mothers can apply a version of the weigh-suckle-weigh technique to assess quantitative transfer of milk to the infant. If this procedure is carried out on a 24 h basis, the daily production of milk can be assessed as was done in a landmark study of secretory activation in the Neville laboratory.[28,29] (3) Finally, the rate of appearance of deuterated water in the saliva or urine of the infant after defined dosage of the mother provides an accurate measure of milk transfer once milk volume has stabilized.[30] While the doses of deuterium necessary are not harmful,[31] it is often difficult to obtain permission to use the technique in mothers who have an aversion to taking an "isotope" (albeit a stable one) while breastfeeding.

The concentration of macronutrients in human milk has been known for many decades.[32] However, recent advances in mass spectroscopic techniques have allowed a remarkable refinement both in the analysis of low abundance proteins and their structures, e.g., glycosylation and phosphorylation, as well as a detailed understanding of the structures of the numerous oligosaccharides present in milk. The importance of these compounds to intestinal microbiome development in the infant is just now being revealed.[33,34]

Analysis of mRNA associated with the MFG is being carried out in a number of species. Most importantly this technique is available for human milk and has been used effectively in the Haymond laboratory at Baylor College of Medicine (Texas) to examine the transcriptome of the human mammary gland during lactation[26] and the gene expression correlates of the onset of lactose synthesis during secretory activation.[35] Application of new technology such as RNA-Seq[36] and advanced bioinformatics technology[37] in situations where lactation is likely to be compromised such as obesity is an exciting prospect for advances in our understanding of human lactation.

Hormonal Control of Anatomical Development

Endocrine and Paracrine Regulation of Ductal Development

The mammary gland is unique since, unlike most secretory organs, the capacity for milk secretion develops in the adult in concert with other reproductive events and is directed by reproductive hormones.[38] The mammary anlagen and the adipose fat pad into which the gland will develop are specified during embryonic development, however, further growth and differentiation must await the hormones of puberty and pregnancy.[39–42] The molecular basis of the developmental process has been best described in the rodent, where the ductal development that occurs during early puberty has been extensively studied using both genetically modified mice and transplantation approaches. Although hormone ablation studies demonstrated a role for estradiol-17β (E2), P4, and PRL in ductal and alveolar development in the 1950s, recent studies have provided better definition of this process and indicate that insulin-like growth factor-1 (IGF-I), growth hormone (GH), and amphiregulin are also important. At birth the gland contains a rudimentary ductal tree that is attached to the teat (Figure 46.1(A)). During puberty the ducts elongate until they reach the margins of the fat pad (Figure 46.1(C)). Elongation of the ducts occurs through proliferation of mammary epithelial cells (MECs) present in bulb-like structures at the ends of the ducts, which are referred to as TEB and disappear by the end of puberty (Figure 46.1(B)). Ductal branching also occurs during puberty resulting in a structure composed of regularly spaced ducts that never cross each other. Spacing between ducts appears to be the result of growth inhibitory effects of transforming growth factor-β (TGFβ).[43] Cross-transplantation experiments by Naylor and Ormandy showed that strain specific patterns of ductal branching in the mouse are a function of the mammary stroma rather than the epithelium,[44] and the extent of branching also varies between different strains of mice. Side branches develop under the influence of P4 during the luteal phase of the estrous cycle. In the rat the side branches are generally more complex and tend to be variable.[45] In the human the lobular units that are derived from the side branches are complex in character. In this species the so-called terminal duct lobular unit (TDLU) consists of a straight extralobular duct that branches into several intralobular ducts terminating in acinar complexes (Figure 46.1(D)).[9,46] In most dairy species, the ductal structure does not reach the margin of the fat pad until the hormones of pregnancy stimulate both ductal elongation and branching.[47]

Estrogens are known to act through two variants of the nuclear estrogen receptor, ERα and ERβ, which are both expressed in the mammary gland.[48] Mice in which expression of ERβ has been eliminated still show normal ductal elongation during puberty.[49] Initial studies demonstrating that ductal elongation was diminished in ERα knockout mice were complicated by the presence of a residual truncated ERα in these mice.[50,51] More recently, elegant studies by Cathrin Brisken and her colleagues have clearly demonstrated a role for ERα in elongation.[52] ERα null MECs transplanted into the cleared fat pad of three-week-old normal mice showed no ductal outgrowth. However, when ERα null MECs were co-transplanted with wild-type MECs, they were able to form ducts, suggesting the presence of a paracrine signaling pathway dependent upon ERα.[52] Further studies

identified amphiregulin as the paracrine growth factor and thereby implicated the epidermal growth factor receptor (EGFR), the only known receptor for amphiregulin, as a critical signaling molecule for ductal elongation.[53] EGFR is expressed in both MECs and the stromal cell compartment, however, only stromal expression of EGFR is required for ductal elongation,[52,54] suggesting the presence of a second paracrine signaling network regulating ductal elongation.

Increased production of circulating GH is coincident with the onset of puberty; formation of TEBs and ductal elongation have clearly been shown to require both GH and its primary transcriptional target IGF-I.[55,56] IGF-I, IGF-II, the IGF-I receptor (IGFIR), and IGF-binding proteins are all expressed in the murine mammary gland throughout puberty.[57] IGF-I is expressed in the mammary gland stroma throughout postnatal development and in the TEBs during puberty.[58] Loss of greater than 50% of IGF-I expression from MECs resulted in a decrease in both ductal complexity and the cyclins A2 and B1, which are important for S and G2 phase progression.[59] The role of circulating versus locally produced IGF-I in ductal elongation has been debated at length; recent studies by Cannata et al. demonstrate that elevated circulating IGF-I can stimulate development resulting in a more complex ductal network.[60] These data suggest roles for both circulating and locally produced IGF-I in pubertal development. However, it is clear that at least a portion of ductal elongation requires local production of IGF-I, which cannot be completely replaced by circulating IGF-I.[61]

In contrast to IGF-I, expression of IGF-II is induced by PRL and not GH.[62,63] During puberty IGF-II is coexpressed with IGF-I, however postpuberty and during early pregnancy IGF-II expression is restricted to ductal and alveolar epithelial cells in a nonuniform pattern reflecting that of the receptors for progesterone (PR) and PRL (PRLR).[64] The differential regulation of IGF-I and IGF-II suggests that they may each have unique roles in mammary gland development. MECs lacking PRLR transplanted into a wild-type mammary fat pad showed normal ductal elongation,[65] suggesting that neither PRL nor IGF-II is necessary for this process.

For a more complete description of these morphological changes the reader is referred to a number of excellent reviews[8,9,45,46] and an excellent online tutorial at http://mammary.nih.gov.

Mammary Development in Pregnancy

The hormones of pregnancy are critical in producing a mammary gland that is capable of producing milk. Although ductal elongation is normal in mice that lack PR, alveolar differentiation does not occur in PR knockout mice, or in wild-type fat pads transplanted with MECs lacking PR.[65,66] Deletion of PR from the stromal compartment does not alter alveolar differentiation during pregnancy. Two different isoforms of PR are produced from different promoters present in the same gene, and analysis of genetically modified mice lacking specific PR isoforms has revealed that only PR-B is required for alveolar development during pregnancy.[67,68]

In addition to P4, PRL is required for lactogenic differentiation during pregnancy. Genetically modified mice lacking either PRL or PRLR have been generated, and ductal elongation is normal in these mice.[69,70] However, analysis of lactogenic differentiation is complicated by the diminished fertility. Alveologenesis and differentiation of PRLR null MECs transplanted into wild-type mammary fat pads is completely blocked.[71] Consistent with a critical role for PRL and its receptor in lactogenic differentiation, MECs lacking either JAK2 or STAT5, the signaling molecules downstream of PRLR are also unable to undergo lactogenic differentiation,[72,73] further demonstrating the critical nature of the PRL pathway in pregnancy-induced mammary gland differentiation.

Understanding the role of IGFs in mammary gland development is confounded by the complex pattern of expression of both IGF-I and IGF-II during pregnancy; however, a role for IGF-I is suggested in recent studies by Sun et al.,[74] who examined lactogenic differentiation in genetically modified mice expressing a dominant-negative form of IGFIR in the mammary epithelium. Expression of this receptor delayed alveolar differentiation, luminal expansion, decreased epithelial cell proliferation, lipid droplet formation, activation of signaling molecules downstream of the IGFIR, and expression of milk protein genes.[74] Despite these dramatic changes, pup growth was not altered, suggesting that either the mammary gland is able to compensate for the observed defects, that complete loss of IGFIR is required, or that the insulin receptor (IR) takes over signaling through these pathways during lactation.[75,76]

In addition to IGFs, there is also increasing evidence that insulin plays an important role in mammary gland development and function in both pregnancy and lactation. The potential importance of insulin and its receptor in mammary gland development and lactation are at variance with previous thoughts that lactation would be insulated from the actions of insulin since insulin levels vary dramatically with food intake.[77] Berlato and Doppler analyzed the expression of both IGFIR and IR during lactation and observed that the levels of the IGFIR decreased at both the mRNA and protein levels following parturition, with an approximately 80% decrease in the amount of mRNA.[76] In contrast, the expression of total IR remained constant at both the mRNA and protein levels.[76] IR exists as the product of two different splice variants, referred to as IR-A and IR-B, which differ in the presence (IR-B) or absence (IR-A) of exon

11, which encodes 12 amino acids that are located at the C-terminus of the extracellular subunit of the receptor.[78,79] The major difference between IR-A and IR-B is that IR-A has a 10-fold higher affinity for IGF-II, making it a second physiological receptor for IGF-II.[78] Analysis of the expression pattern of IR-A and IR-B during pregnancy and lactation revealed that while the mRNA levels of IR-A remain the same over this developmental time course, the amount of IR-B mRNA increases three- to fourfold following parturition.[76] When the sixfold decrease in the amount of IGFIR mRNA that occurs following parturition is also taken into account,[76] one must conclude that there is a dramatic increase in insulin signaling during lactation.

The importance of insulin signaling in mammary gland development and lactation is indicated by recent analysis of mice bearing a floxed IR. Excision of the IR gene from MECs using Cre recombinase expressed specifically in these cells resulted in the formation of mammary glands that lack at least 50% of secretory alveoli by day 14 of pregnancy, and in a 75% decrease in pup growth over the first 10 days of lactation.[80] Despite this dramatic decrease in pup growth, the majority of pups do survive. A comparison of the genetically modified mice expressing the dominant-negative form of IGFIR and mice lacking expression of IR in the mammary epithelium clearly provides evidence that while IGF-I may be important for alveolar development during pregnancy, it is not the entire story in either pregnancy or lactation. These observations suggest that conditions that impair insulin signaling, such as obesity and diabetes, may have dramatic effects upon lactation.

Functional Differentiation

Functional differentiation of the gland can be divided into three phases that have been most thoroughly studied in the mouse: (1) the *proliferative* phase of early pregnancy, (2) *secretory differentiation* starting in mid-pregnancy, during which the gland becomes competent to secrete milk, and (3) *secretory activation* around parturition when the secretion of milk commences.

The Proliferative Phase

This phase starts immediately after conception, reaching a peak about day 5 of pregnancy, when an astonishing 25% of the mammary alveolar cells are labeled with ^{3}H-thymidine 1 h after injection in the mouse (Figure 46.3(A)).[81,82] Cell proliferation tapers off gradually through the remainder of pregnancy until MECs reach quiescence just prior to parturition. During this period the content of mRNA for epithelial markers cytokeratin 19 and claudin 7 increases nearly three orders of magnitude (Figure 46.3(B)),[83] indicating a remarkable expansion of the alveolar compartment. Whether these markers reflect cell number, cell size, or a denser cytoplasm is not currently clear. Three paracrine factors—Rank ligand (RANKL), WNT 4, and amphiregulin—show an expression pattern that parallels proliferative activity (Figure 46.3(C)); expression of RankL is regulated by PR[68] while expression of amphiregulin is induced by IGF-II.[62,63] In the mouse a single, coordinated round of cell division takes place immediately after parturition (Figure 46.3(D)),[82] possibly resulting in the high proportion of binucleate cells present in the lactating gland[85]; it is currently unknown what growth factors stimulate this round of cell division as the three paracrine factors elevated during early pregnancy are not expressed at this time. It is also not clear whether the proliferative activity in early lactation is specific to the mouse or occurs in other species as well.

As mentioned before, studies using genetically modified mice have indicated that P4[86,87] and PRL[88] are the major hormones that promote side branching and the formation of alveoli.[39] It is surprising to note that the receptors for PR are expressed in only a subset of cells in the mammary epithelium[64,89–92] (Figure 46.4). Indirect evidence indicates that this is also the case for the PRLR. Analysis of normal human mammary epithelium has shown that 98% of the proliferating cells are negative for estrogen receptor (ER) and PR,[90] and, significantly, the absence of PR in proliferating cells has also been observed in the mammary epithelium of mice, rats, and cows.[94–96] The disparate localization of proliferating cells and PR^{+} epithelial cells could be explained by the rapid downregulation of steroid receptors following ligand binding; this hypothesis is supported by observations that ERα is rapidly degraded by the proteasome,[97] and that ERα is also rapidly lost in MECs following entry into the cell cycle.[98]

The generally accepted view is that PR is not expressed in proliferating cells and that P4 action induces expression of paracrine growth factors that stimulate proliferation of PR^{-} epithelial cells. Thus, PR has been shown to induce expression of RANKL[68] and WNT-4,[99] while PRL induces expression of IGF-II.[62,63] WNTs are a family of signaling molecules similar to the *Drosophila* protein wingless and the mammalian INT. Overexpression of WNT-4 stimulates ductal branching and formation of alveoli following transplantation into cleared mammary fat pads in a manner that resembles development observed in pregnant mice.[100] Further studies have demonstrated that expression of WNT-4 is dependent upon activation of PR by P4, and that WNT-4 functions in a paracrine manner to induce ductal branching and alveologenesis during pregnancy.[99] It is not clear whether WNT-4 acts directly upon PR-negative MECs, or on other cells in the surrounding microenvironment.

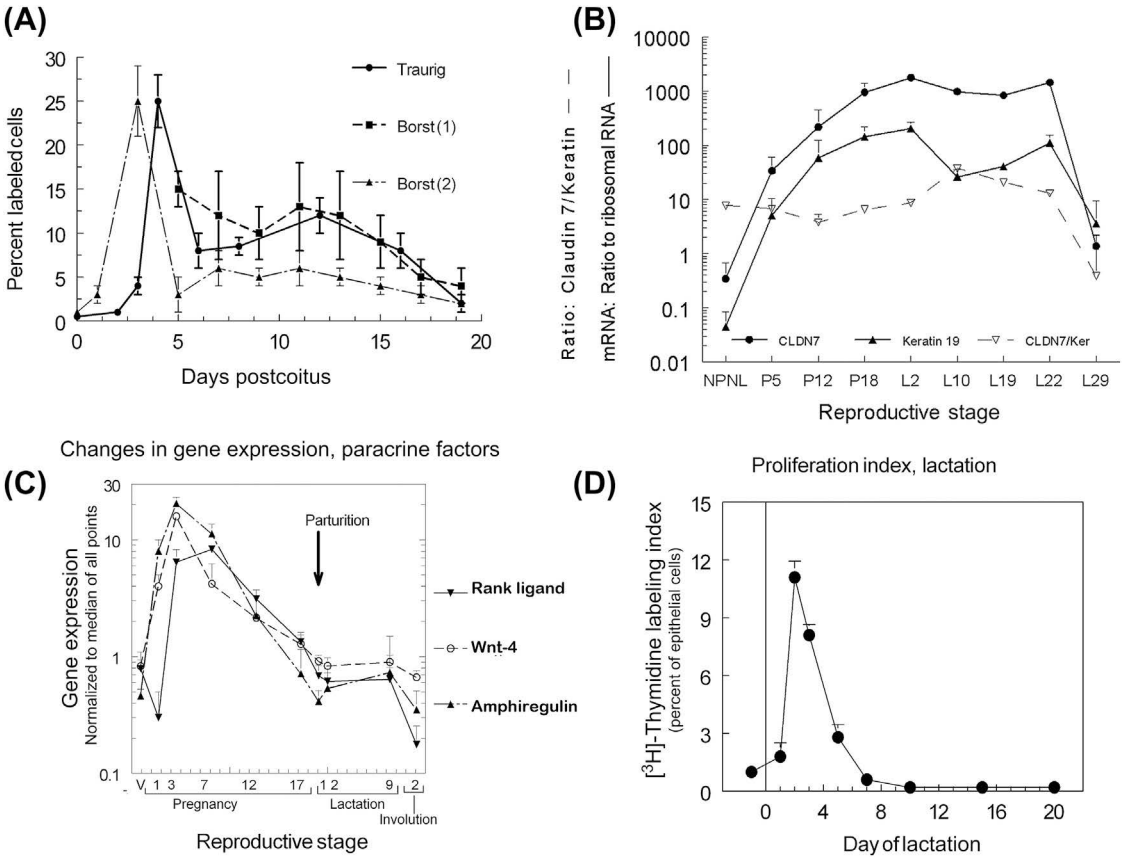

FIGURE 46.3 Proliferative activity during pregnancy and lactation in the mouse. (A) Proliferation through pregnancy as measured by 1 h incorporation of ^{3}H-thymidine in vivo. (B) Expression of mRNA for keratin 19 and claudin 7, epithelial cell markers determined by real time RT-PCR. Dashed line is the ratio of claudin 7/keratin 19. These changes are indicative of the proportion of epithelial cells in the gland as pregnancy progresses. (C) Changes in expression of mRNA of the paracrine factors Rank ligand (RANKL), Wnt-4, and Amphiregulin over pregnancy and lactation. (D) One hour ^{3}H-thymidine labeling index during lactation. The synchronized round of DNA synthesis probably results in the production of the binucleate cells that are numerous in the mammary gland of the lactating mouse. *Source: (A) Data replotted from Refs 81,82; and Borst DW, Mahoney WB. Mouse mammary gland DNA synthesis during pregnancy.* J Exp Zool *1982;**22**:245–50. (B) Reproduced from Ref. 83. (C) Unpublished data from Ref. 84. (D) Data replotted from Ref. 82.*

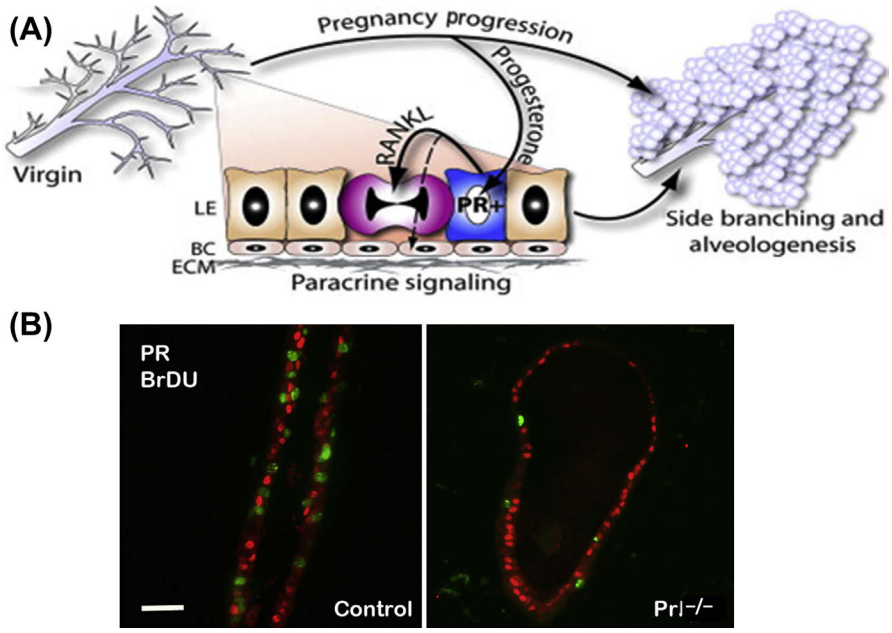

FIGURE 46.4 Role of paracrine signaling in alveologenesis. This figure is reproduced in color in the color plate section. (A) As pregnancy progresses side branches develop at discreet intervals along the ducts; cells in these side branches proliferate to form alveoli. The progesterone receptor (PR) is expressed in a subset of alveolar cells that are stimulated by increasing P4 from the ovaries to secrete RANKL. This paracrine factor acts on neighboring cells to promote proliferation. (B) Expression of PR and proliferating cells after 2 days of treatment with E2 and P4 in wild-type (control) and PRL-null mice. Proliferating cells (BrDU labeled, green) and PR positive cells (red) are generally not coincident. Note that the number of proliferating cells is reduced in the absence of PRL. Scale bar, 50 μm. *Source: (A) Used by permission from Ref. 93. (B) Original figure from Ref. 64.*

To observe the effects of overexpression of RANKL in MECs, Fernandez-Valdivia et al.[101] constructed transgenic mice in which expression of RANKL was driven by the mouse mammary tumor virus (MMTV) promoter, resulting in RANKL expression in the pubertal mammary gland when RANKL is not normally expressed. Transgenic mice expressing RANKL exhibited more TEBs, ductal side branching, and alveolar buds reminiscent of an early pregnant mammary gland, and there was a dramatic increase in the number of proliferating cells in both ductal and alveolar structures.[101] The florid proliferation and development observed in the RANKL transgenic mice is consistent with the attenuated alveologenesis and lactation defect observed in the RANKL knockout mice.[102] Expression of RANKL in MECs in vivo can be induced by treatment with either P4 or PRL.[62,67] Induction of RANKL is restricted to PR^+ cells, which reside in close proximity to PR^- cells that express cyclin D1 and proliferate in response to P4.[68] Further details regarding both WNT-4 and RANKL can be found in excellent recent reviews by Rajaram and Brisken[103] and Fernandez-Valdivia and Lydon.[93] Future research is needed to reveal the cells targeted by WNT-4, the signaling pathway(s) activated by WNT-4, and the role of these ligands in maintaining mammary stem cells in the normal gland. How amphiregulin, whose expression pattern in early pregnancy parallels that of WNT-4 and RANKL (Figure 46.3(C)), fits into this picture is not yet clear.

Another interpretation comes from experiments in the Haslam laboratory where the mammary gland from early pregnant mice showed a decrease in expression of PR-A, while the expression of PR-B remained constant in alveolar epithelial cells. Further, and in contrast to the virgin gland, expression of PR-B co-localized with cells that label with 5-bromo-2′-deoxyuridine (BrDU) indicating that these cells are proliferating.[104,105] These authors concluded that P4 interacting with PR receptor directly stimulated proliferation of alveolar cells during pregnancy.

Secretory Differentiation

Secretory differentiation, previously referred to as lactogenesis I,[106] begins about mid-pregnancy and is signaled by changes that depend on the species and the experimental paradigm used for their study. For example, Akers[47] showed that α-lactalbumin increased in mid-pregnancy in the fluid extracted at this time from the bovine mammary gland; a similar phenomenon is also observed in the mouse. McManaman and colleagues[16,107] have documented the accumulation of lipid droplets in the mammary alveolar cells of the mouse starting around day 8–10 of pregnancy (Figure 46.5(A)). Mellenberger and Bauman[108] showed a biphasic increase in the enzymes of lipid synthesis in the rabbit mammary gland, and Hartmann and colleagues[109] showed that lactose appeared in the plasma and urine in the women at mid-pregnancy. Gene expression profiling of whole murine mammary glands during pregnancy and lactation has revealed that expression of milk protein genes increases about fivefold during pregnancy and then increases a second time at parturition (Figure 46.5(B)).[84] Similar data are obtained when the expression of these genes is examined in preparations of adipocyte-depleted MECs (Rudolph and Anderson, unpublished data). Analysis of genes involved in de novo synthesis of fatty acids and β-oxidation of fatty acids reveals that the latter decrease over the course of pregnancy, while expression of genes involved in fatty acid biosynthesis increases sharply at parturition. Expression analysis of adipose-depleted MECs reveals that the increase in fatty acid biosynthetic genes is specific to the epithelium during pregnancy.[19] There is also a decrease in the proportion of adipose tissue observed in the mammary gland by microscopic examination, reflected by a proportionate decrease in expression of adipocyte-specific mRNAs. However, this decrease may reflect expansion of epithelial cells rather than loss of adipocytes (Figure 46.5(A)).[84] There is also a decrease in collagens expressed in the mammary gland as pregnancy progresses; it is not clear whether this change represents stromal remodeling or simply dilution of stromal components by the expanding epithelium. During late pregnancy the gland begins to produce small amounts of secretion product. This product can escape the gland through the junctional complexes between the alveolar cells, which are highly permeable during pregnancy.[110,111] Copious milk secretion in all species that have been examined is inhibited by the high concentrations of circulating P4 produced by the ovaries or placenta, depending on species, at this time.

Hormonal Regulation of Secretory Differentiation

PRL, P4, and the lactogenic product of the placenta, placental lactogen (PL), have all been implicated in the regulation of secretory differentiation.[112] There are clearly species differences in the roles of these hormones, particularly PRL and PL, and even though genetically modified mice have allowed a good understanding of the role of PRL, the role of PL remains unclear. GH is lactogenic in many species,[113] but its role in differentiation has been controversial because it is not clear whether the growth hormone receptor (GHR) is expressed in stromal cells or epithelial cells.[114] Lactation occurs although it is not normal in the $GHR^{-/-}$ mouse.[115] Furthermore, definition of the precise role of GH is complicated by its ability to induce expression of IGF-I, which has been shown to have direct effects upon secretory differentiation (see previous discussion).

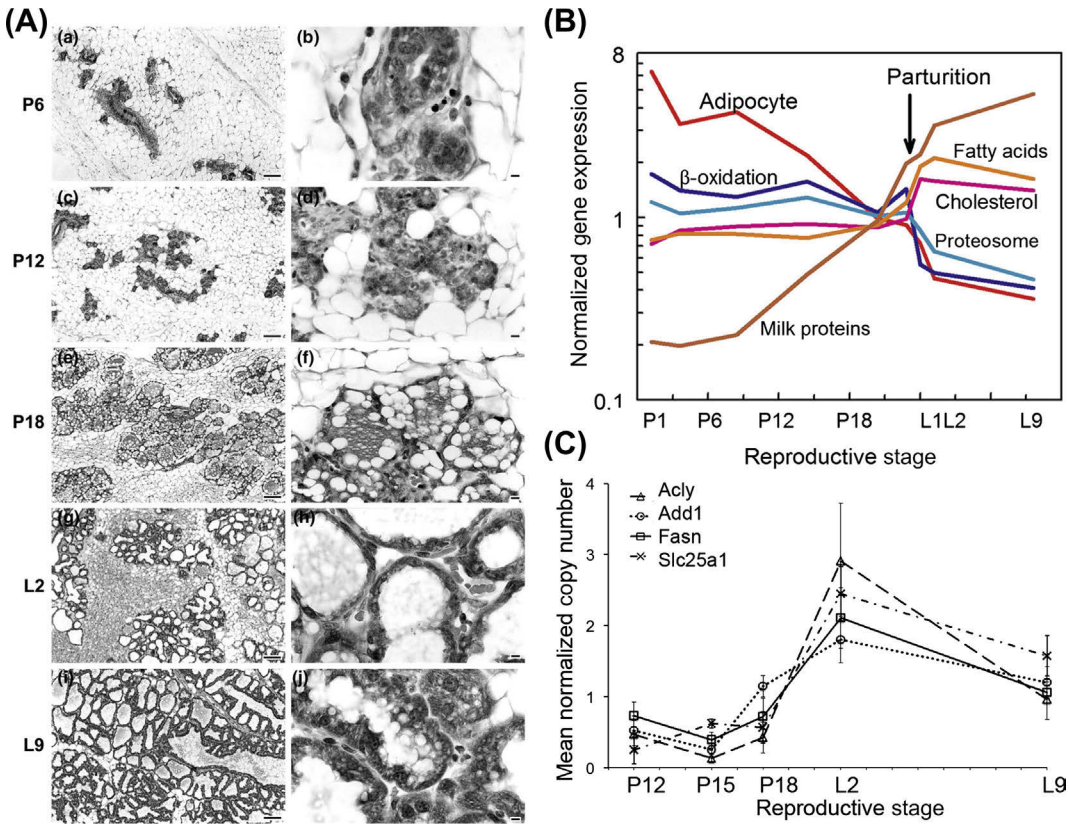

FIGURE 46.5 Changes in the differentiated activity of the mammary gland in pregnancy and lactation. Part B of this figure is reproduced in color in the color plate section. (A) Morphology of secretory development in the mouse. Shown are histological sections of the mammary gland of FVB mice through pregnancy and lactation. Mammary glands were isolated on the days indicated, fixed, sectioned, and stained with hematoxylin and Eosin. Scale bars, 100 μm in (a, c, e, g, and i) and 10 μm in (b, d, f, h, and j). Note the intracellular lipid droplets in late pregnancy (panel (f)), the expansion of the lumens at the onset of lactation (panels (f) and (h)), and the diminution of adipocytes as lactation progresses (compare panel L2 and L9). (B) Changes in gene expression of different categories of genes in the mouse mammary gland from microarray studies. Expression of adipocyte-specific genes and collagens (not shown) decreases six- to eightfold during pregnancy and another twofold at parturition, whereas the genes for fatty acid degradation and many components of the proteosome are level during pregnancy and decrease about twofold at parturition. Milk protein genes, on average, increase about fivefold during pregnancy and another threefold around parturition, whereas the genes for fatty acid and cholesterol synthetic enzymes increase about twofold just after parturition. Normalized data for each class were averaged to produce the lines on this graph. (C) Time course of expression of genes important for de novo lipogenesis. Note that the major increase in expression occurs during secretory activation. *Source: (A, B) From Ref. 11. (C) Original data from Rudolph MC. University of Colorado, Denver.*

PROLACTIN

PRL remains high throughout human pregnancy, and women with low PRL levels during pregnancy have difficulty in lactating,[116] suggesting that PRL is involved in secretory differentiation in humans.[117] The role of PRL in secretory differentiation has been more extensively studied in rodents, and although PRL is secreted in a pulsatile fashion early in pregnancy (Figure 46.6(A)), PRL itself is not necessary in mid-pregnancy in rodents and may even be deleterious by inducing premature secretory activation. Circulating PRL returns to high levels just prior to parturition[119] (Figure 46.6(B)). Furthermore, expression of the PRLR changes during pregnancy, and the long form, which activates downstream signaling pathways, is downregulated in pregnancy and increases markedly at parturition[118] (Figure 46.6(D)). Finally, if a constitutively active PRL receptor is expressed under the control of the β-lactoglobulin promoter, alveolar development is increased in late pregnancy, but the animals fail to lactate.[120] PRLR and the downstream signaling molecules JAK2 and STAT5 are required for secretory differentiation since mice lacking PRLR, JAK2, or STAT5 lack expression of milk protein genes.[69,72,73,121,122] PRLR, like PR, is expressed in only a subset of epithelial cells in early pregnancy,[64,123] and it is not yet clear whether these cells are coincident or not.

In the human serum, PRL and PL levels rise starting in early pregnancy reaching the very high levels of about 100 ng/ml (compared to ~10 ng/ml in the nonpregnant woman) and 2000 ng/ml (nonexistant in the nonpregnant animal), respectively, by the twentieth week of gestation; increases in PRL levels directly correspond to increases in lactose excreted into urine,[124,125] implying that PRL regulates activation of lactose synthesis.

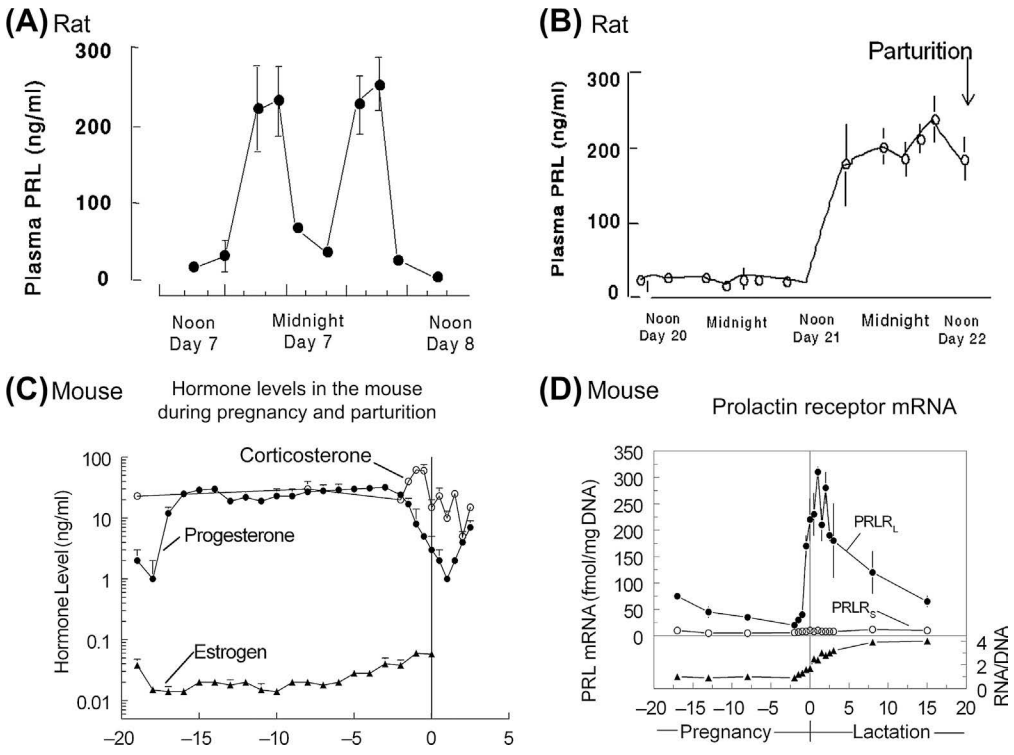

FIGURE 46.6 Hormone profiles during pregnancy in rats and mice. (A) Pulsatile secretion of PRL during early pregnancy in the rat. At 8 days postcoitus, this pulsatile activity ceases. (B) Return of PRL secretion 24h prior to parturition in the rat. (C) Steroid hormone profiles in the mouse during pregnancy. Corticosterone remains approximately constant with a slight elevation at parturition, possibly due to stress. P4 rises early in pregnancy and falls one day prior to parturition. Estradiol rises about threefold over the course of pregnancy. (D) Expression of mRNA for the long (PRLL) and short (PRLS) forms of the prolactin receptor during the transition from pregnancy to lactation. Note the increase in the ratio of total RNA to DNA at the onset of lactation. *Source: (A, B) Modified from Ref. 118. (C, D) Data replotted from Mizoguchi Y, et al. Corticosterone is required for the prolactin receptor gene expression in the late pregnant mouse mammary gland.* Mol Cell Endocrinol *1997;**132**:177–83.*

PLACENTAL LACTOGEN

PL is present at high levels only during secretory differentiation.[126–129] The hormone has evolved at least twice, in rodents and ruminants from the PRL gene and in primates from the GH gene.[112,130,131] Women with deletions in the GH/PL gene complex and undetectable PL concentrations in plasma have been reported to have quite normal lactations,[132,133] suggesting that PRL is the hormone responsible for secretory differentiation in humans. Several rodent PLs have been fully characterized (mouse, rat, hamster) and their patterns of secretion in pregnancy measured. The twice-daily surges of pituitary PRL in rats are replaced in mid-pregnancy by, first, a surge in PL I and then by PL II, which continue to increase until term. There is currently no evidence for a specific PL receptor, and PL is thought to act through either the GHR, the PRLR, or both.[134]

PROGESTERONE

P4 increases early in pregnancy (Figure 46.6(C)) secreted by the ovaries. As discussed previously, there is good evidence that it drives proliferation during early pregnancy; however, it is not clear whether it plays a role in secretory differentiation during mid- to late pregnancy. In most species ovarian secretion of P4 continues through pregnancy. However, in women ovarian P4 is replaced by placental P4 after the first trimester. There is incontrovertible evidence that it does inhibit secretory activation in late pregnancy[135,136] and prevents premature lactation in all species that have been studied. P4 withdrawal triggers both secretion and tight junction closure. Thus these processes are triggered in the mammary epithelium of late pregnant mice by injection of the PR antagonist RU486; PRL and glucocorticoids are required.[136] This observation, which has been repeated in many species, indicates that P4 is able to suppress secretory activation through the small number of P4 receptors present in late pregnancy, although it is not clear whether an unidentified paracrine factor could be involved in a manner similar to that responsible for alveolar proliferation in early pregnancy or whether myoepithelial cells could be involved.

Secretory Activation

Secretory activation is the onset of copious milk secretion; it is sometimes referred to as lactogenesis II or the initiation of lactation.[106] As described before, this process is set in motion by the fall of P4 around parturition in all species that have been studied[135,137–142]; while in mice the fall in P4 occurs at parturition, in humans P4 levels fall two orders of magnitude over the first four days postpartum, resulting in the production of approximately 500 ml/day of milk by day 5.[143] In both the mouse and human, PRL levels are high at parturition. In the mouse the fall in P4 is accompanied by profound changes in the expression and metabolic activities of many classes of molecules including milk proteins and the enzymes of lipid and cholesterol synthesis (Figure 46.5(B)). The expression of milk protein genes is largely regulated by PRL via the PRLR/JAK2/STAT5 signaling pathway due

to the presence of specific promoters that bind STAT5,[14] however, the regulation of fatty acid biosynthesis is less well defined.

Among potential regulators of fatty acid biosynthesis are PRL, sterol-response element binding protein (SREBP), AKT1, and SPOT14 (also known as THRSP). Experimental evidence supports roles for both SREBP and PRL in regulating enzymes involved in fatty acid and cholesterol biosynthesis[19,20] (Figure 46.7). Analysis of gene expression profiles in adipose-depleted MECs from lactating mice that were treated with bromocriptine for 8 h to block PRL-dependent signaling demonstrated a two- to fourfold decrease in the expression of genes involved in glycolysis and the pentose shunt, a three- to sixfold decrease of genes encoding enzymes involved in de novo fatty acid biosynthesis, and a two- to twentyfold decrease in the expression of genes involved in triacylglycerol synthesis.[20] When SCAP was deleted from MECs, SREBP function was lost and pup weight gain decreased from day four of lactation on. Expression of fatty acid synthase (FASN), the mitochondrial citrate transporter SLC25A1, and stearoyl-CoA-desaturase (SCD2) were all decreased in MECs from lactating mice lacking SCAP, however, the levels of other enzymes involved in de novo fatty acid biosynthesis were unchanged.[19] These findings indicate that PRL has a more significant role in upregulating the expression of enzymes involved in de novo fatty acid biosynthesis at secretory activation than SREBP, which regulates this process in the liver and adipose tissue. However, both molecules do contribute to the regulation of these genes (Figure 46.7). It is very likely that other levels of control remain to be discovered as de novo fatty acid biosynthetic enzymes are likely to be regulated at both the

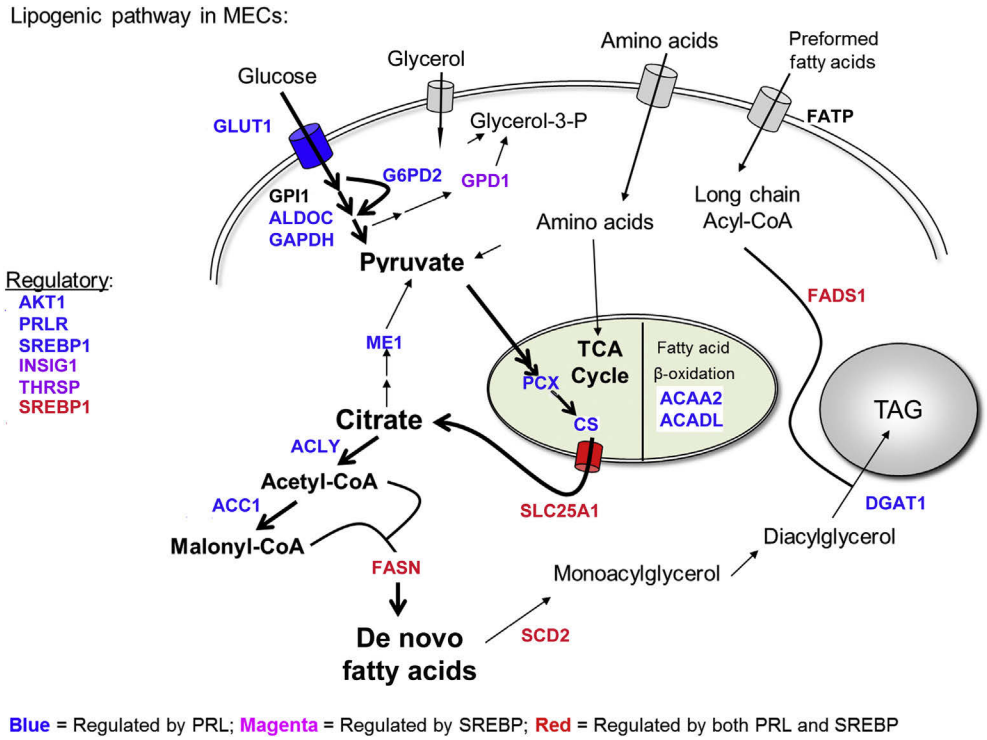

FIGURE 46.7 Lipid synthesis pathways in mammary epithelial cells during lactation. This figure is reproduced in color in the color plate section. Substrates for lipid synthesis enter the cells via the glucose transporter (GLUT1), a glycerol transporter, as amino acids, or as preformed fatty acids via a fatty acid transport protein (FATP). Glycolysis leads to the production of both glycerol-3-phosphate and pyruvate from glucose. The genes for several enzymes in this pathway, fructose bis-phosphate aldolase (ALDOC) and glycerol-3-phosphate dehydrogenase (GAPDH), as well as glycose-6-phosphate dehydrogenase (G6PD2 in the mammary gland) are all upregulated by prolactin along with mitochondrial genes for pyruvate carboxylase (PCX) and citrate synthase (CS). Glycerol-3-phosphate is formed from dihydroxyacetone phosphate, a product of glycolysis, by glycerol-3-phosphate dehydrogenase (GPD1) to be used as a backbone for triacylglyceride (TAG) synthesis. GPD1 is regulated by SREBP. Amino acids are transformed into pyruvate and other substrates that enter the mitochondria to be transformed into citrate, which is exported via the tricarboxylic acid transporter, SLC25A1. Citrate is the major substrate for de novo synthesis of fatty acids in species other than ruminants, which utilize acetate for this purpose. Citrate is transformed into acetyl-CoA by ATP citrate lyase (ACLY), then to malonyl CoA by acetyl-CoA carboxylase (ACC1), and finally to saturated fatty acids with 8–16 carbons (C:8–C:16) by fatty acid synthase (FASN). Cytosolic malic enzyme (ME1) and the enzymes of the pentose phosphate shunt both provide the necessary reducing molecule NADPH that is required for activity of FASN. C:16 fatty acids can be desaturated by stearoyl-CoA desaturase (SCD2) prior to being esterified into monoacylglycerol, then diacylglycerol, and finally into triacylglycerols, with subsequent integration of the fatty acids derived from preformed sources. The final step in the TAG synthesis pathway is catalyzed by diglyceride acyltransferase (DGAT1). The TAG coalesce into lipid droplets. Both prolactin and SREBP have been shown to regulate the genes for FASN, SLC25A1, SCD2, and FADS1. *Source: Diagram derived from data in Refs 19,20.*

posttranscriptional and posttranslational level by diet and other factors.

Lactose is the main osmotic agent present in milk, and the levels of lactose are a major determinant of the amount of water drawn into milk in most species. The synthesis of lactose in the mammary gland requires two proteins present in the Golgi compartment, galactosyltransferase and α-lactalbumin; these combine to produce an enzyme capable of producing lactose at physiological concentrations of glucose.[144] It should be noted, however, that milk from fur seals, sea lions, and walruses does not contain lactose apparently because of a mutation in their α-lactalbumin gene.[145] It has also been suggested that the presence of this mutated form of α-lactalbumin may allow the mammary glands of these species to maintain lactation even in the presence of long interruptions that occur during foraging.[145] Synthesis of lactose has been examined in rodents, dairy cows, and humans,[134,144,146–150] and it is clear that P4 inhibits high level synthesis of lactose during pregnancy. Thus robust expression of the requisite enzymes and high level synthesis/secretion occurs around parturition, the timing depending on the timing of the fall in P4.

Lactation

The continuous production of milk is known as lactation; in earlier literature it was referred to as galactopoiesis. As noted before, lactation is set into motion by a decrease in serum levels of P4 and either a rise in PRL levels (rodents) or maintained high PRL levels (humans). In rodents and dairy animals the decrease in P4 occurs the day before parturition, also setting parturition in motion. In humans, where most P4 is made by the placenta, this decrease takes place over four days after removal of the placenta. Levels of PRL rise at parturition in rodents (Figure 46.6(B)), coincident with a rise in the PRL receptor (Figure 46.6(D)). Further rises in humans and dairy animals after parturition are dependent on stimulation by suckling.[124,151,152] Once lactation is initiated, it is sustained by two different hormones, PRL and OT.[151] During lactation, PRL secretion is stimulated by suckling or milking; in seasonal animals the plasma concentration of PRL is influenced by the day length,[153] the time since feeding/eating, and the time postpartum (reviewed in Ref. 154). Treatment of women,[155] rats,[156] and mice[20] with bromocriptine, a dopamine analog that suppresses PRL release from the pituitary, blocks lactation. In addition to being required for expression of milk protein genes, studies with bromocriptine demonstrate that PRL is required for expression of genes involved in glucose metabolism and de novo synthesis of fatty acids. Furthermore, loss of PRL results in apoptosis of MECs, indicating that it activates survival pathways. Circulating levels of PRL generally decrease as lactation proceeds[96,157] despite continued high production of milk, providing evidence that PRL does not control day-to-day milk volume production.

As described in further detail in the sections Conclusion Milk Ejection, OT is critical during lactation as it stimulates the contraction of myoepithelial cells resulting in the ejection of milk from the alveolus into the ducts. If this function is impeded in any way, involution ensues.

Lactation consists of two phases in placental mammals, a colostral phase during which the secretion product contains large concentrations of immunoglobulins and other protective substances[158] and a mature secretion phase during which large quantities of milk are produced. Colostrum is critical in species such as dairy animals, which do not transport immunoglobulins across the placenta. In these species colostrum contains particularly large quantities of immunoglobulins, which are transported across the intestinal epithelium, providing immunoprotection to the young, which suffices until maturation of their own immune systems.[158] It has been suggested that the delay in the production of mature milk in humans is beneficial in exposing the infant to the immune-protective components of colostrum,[159] which is, however, produced in very small quantities. Mature milk provides all the nutrients required by the offspring of the particular species for growth during a period of time that varies from 7 days in the guinea pig to 4 to 6 months in the human. Some adjustments in milk composition occur during this process.[28] For example, in humans the concentrations of protein, Na^+, K^+, Cl^-, and Ca^{++} decline significantly between 3 weeks and 6 months postpartum, whereas lipid, lactose, and glucose increase.[28] In general these changes are small, usually not more than 20%.

When both the concentration of milk constituents and milk volume were monitored in 12 very cooperative women, a dramatic illustration of the changes in the activity of the gland over the period of secretory activation was obtained (Figure 46.8).[29,160,161] The increase in milk volume, which takes place primarily between days 2 and 4 postpartum in women and earlier in many other species,[29,160–162] is brought about by the coordinated increase in the activity of pathways for ion (citrate, $PO_4^=$, K^+, Mg^{++}, Ca^{++}, Na^+ and Cl^+) secretion and for lipid, lactose, and protein synthesis and secretion. A number of other changes take place prior to this coordinated increase in milk secretion. As detailed in the section on cell–cell interactions, although present between alveolar cells throughout pregnancy, tight junctions are highly permeable, closing only at the onset of lactation, leading to a decrease in the Na^+ and Cl^- flux from the interstitial space into the lumen as a well as a decrease in lactose flux out into the interstitium; the result is a rapid increase in lactose concentration and fall in milk Na^+ and Cl^-.

One of the remarkable aspects of lactation is that milk production can expand to meet increased demands, and

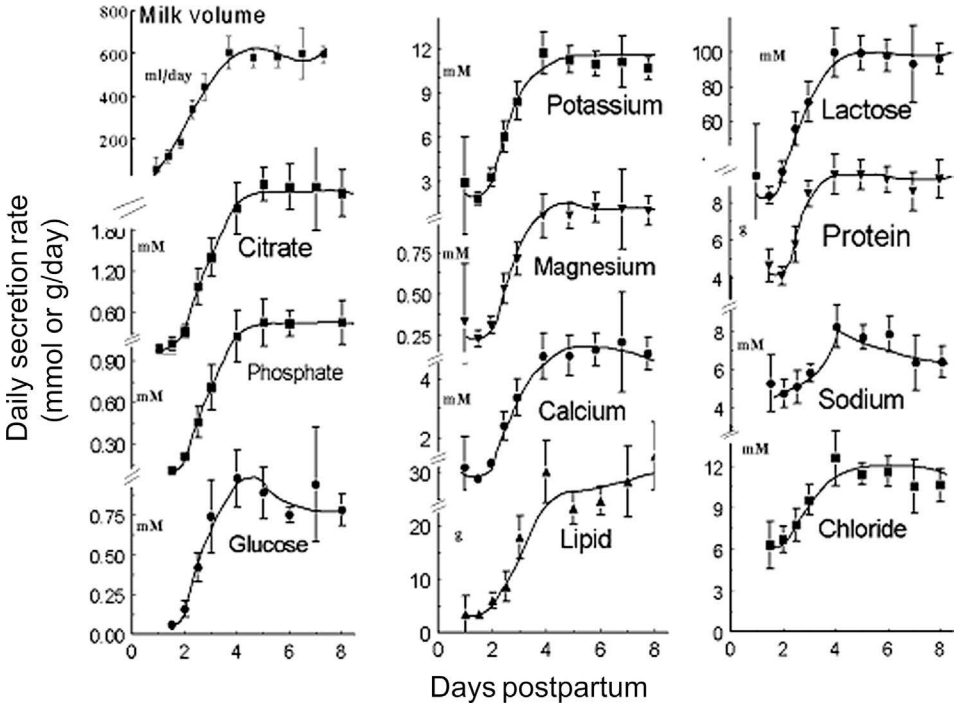

FIGURE 46.8 Milk volume secretion and the rate of secretion of several milk components during the first week postpartum in women. Twelve subjects weighed their infants before and after every feed for the first week postpartum and mid-feed milk samples were taken twice a day from each breast. *Source: Reproduced by permission from Ref. 160.*

can cease when milk is no longer needed. The precise feedback mechanisms that control the dynamics of lactation have been the topic of great speculation for a long time, specifically around the topic of what are the sensors for milk stasis that lead to loss of milk secretion. The long hypothesized "feedback inhibitor of lactation"[163] has never been purified or proven to exist, however, in recent years attention has focused on the possibility that serotonin may play this role. While searching for PRL-induced transcripts, Matsuda et al. identified the enzyme tryptophan hydroxylase (TPH) as a target for PRL that was induced during pregnancy and lactation.[164] TPH is the rate-limiting enzyme in the synthesis of serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter regulating mood and cognition. Expression of TPH was induced by PRL in a time- and concentration-dependent manner, and its expression was highest in mammary glands whose teats were sealed producing milk stasis. Further studies showed that treatment of mammary tissue slices or MECs with 5-HT suppressed expression of milk protein genes and induced histology similar to that of involuting mammary glands.[164] Further study demonstrated that 5-HT regulated tight junctions and milk secretion,[165] suggesting that accumulation of 5-HT in the interstitial fluid surrounding secretory alveoli during milk stasis could lead both to cessation of milk secretion and apoptosis of epithelial cells. Treatment of bovine MECs and lactating cows with the selective 5-HT uptake inhibitors (SSRI) such as fluoxetine increased plasma lactose and increased the ratio of Na^+/K^+ in the milk, decreased expression of milk protein genes, and decreased milk volume secretion in lactating cows.[166] These studies were confirmed in mice; further, Tph1 (tryptophan hydroxylase 1) null mice were resistant to these effects.[167] SSRIs are a highly prescribed class of drugs that are commonly used to treat depression, and therefore it should be of little surprise that pregnant women taking SSRIs were found to be more likely to experience delayed secretory activation.[167] It has been long appreciated that there is a delay in the onset of lactation in obese women, and recent studies have suggested that a high fat (HF) diet significantly increases the expression of TPH1 and the mammary receptor for 5-HT (HTR7).[168] These findings produce tantalizing evidence that the serotonin pathway may contribute to the effects of diet and obesity upon lactation.

For a relatively complete description of milk composition in various species, see works by Jenness[169,170] and Jensen.[171] In marsupials, in which the young are born at an extremely immature stage and develop within an abdominal pouch, the changes in milk composition during lactation are much more extreme.[172] A new area of investigation is how the composition of milk from primates may change with the gender of the infant, as well as psychological/emotional factors,[173–176] raising fascinating questions in evolutionary biology.

Involution

After weaning, most of the secretory cells as well as their stromal environment undergo a process of remodeling, and the mammary gland returns to a state similar

to that of the virgin through a process referred to as involution.[177,178] This process is complex, highly regulated, and occurs in two distinct phases: the first phase is reversible and protease independent, while the second phase is not reversible, depends on expression of proteases, and involves considerable remodeling of connective tissue.[179] The use of genetically modified mice and global gene expression profiling over the last decade has greatly advanced our understanding of the changes that occur during the first six days of involution. The vast majority of these studies have used a model of forced involution in which the pups are removed from the lactating dam on day ten of lactation. Although this model is artificial when compared to normal weaning, which is more gradual, it does allow for a very reproducible series of events, which have been well characterized at the cellular and molecular levels. An alternative approach involves sealing of teats on one or more glands to induce milk stasis and involution in the affected gland. This less utilized approach has the advantage that functional and nonfunctional glands can be compared in the same animal with the maintenance of the hormonal environment of lactation.

The original definition of the two phases described the time, approximately 48 h after pup removal, at which the process becomes irreversible: if pups are returned to the dam within 48 h, lactation resumes.[179] In the human this period is about three days. Interestingly, there are species such as fur seals in which lactation can be interrupted by at least 3 weeks when the mothers feed offshore before returning to nurse.[180] A more functional definition might divide the 6-day involution interval into the period, usually 3 days in the rodent, during which alveolar volume is markedly decreased by removal of both milk and epithelium,[181] and a second phase starting after 3 days that involves stromal remodeling. Teat-sealing studies in the mouse clearly demonstrated that the first phase is regulated by local factors within the individual gland and not by circulating hormones.[179,182]

During the first day after pup removal the luminal spaces fill with milk (Figure 46.9); by 24 h shed, apoptotic cells appear within this expanded luminal space. It has been suggested that MECs die of an apoptotic process since activation of executioner caspases (caspase 3 and 8) and fragmentation of DNA typically associated with this process are readily detected.[184–187] However, the shed "apoptotic" cells do not have the classical appearance of apoptotic cells in other organs in that they are swollen, frequently have two hypercondensed regions of chromatin, and lack the membrane blebbing classically associated with apoptotic cells.[188] Monks et al.,[181] in an elegant study that for the first time took into account the volume of the gland, found that the peak of apoptosis occurs between 2.5 and 3 days following pup withdrawal and that almost all of the apoptotic cells as well as the milk are cleared in this interval. In this study MECs were thought to be responsible for the removal of apoptotic bodies because they did not detect markers of macrophages in the mammary gland during the first 3 days following pup withdrawal.[181] These authors

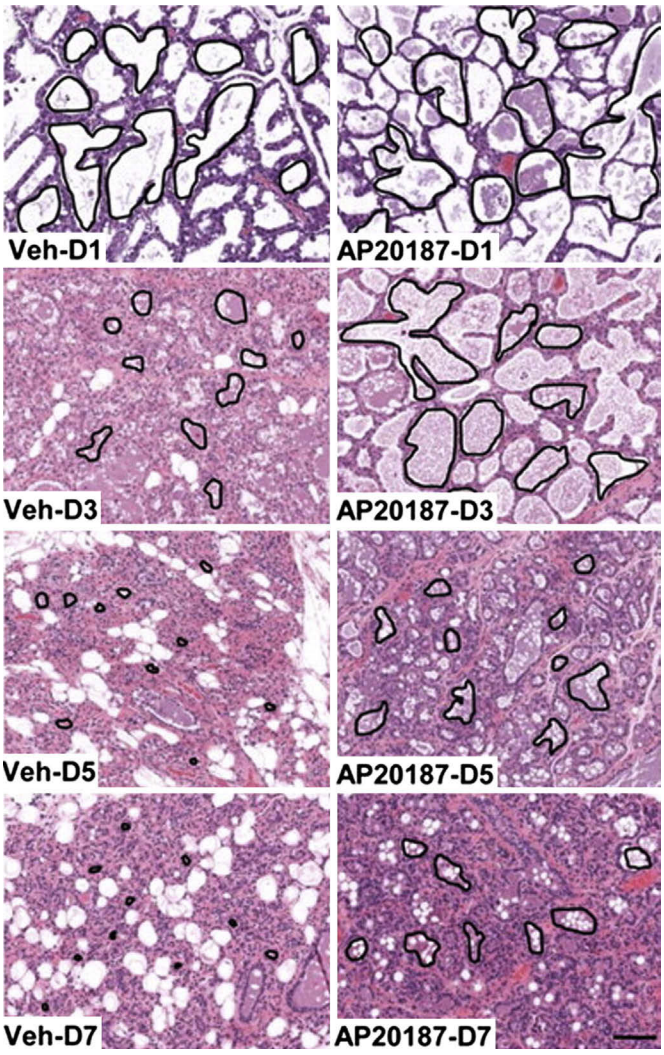

FIGURE 46.9 The role of macrophages in mouse mammary gland involution; histological analysis. This figure is reproduced in color in the color plate section. Glands from Mafia mice were analyzed on days 1, 3, 5, and 7 after pup removal from a 10-day lactating mouse suckling five to six pups. Three days prior to pup withdrawal experimental dams were given a dose of AP20187, which depletes macrophages in this strain. Left-hand images, vehicle only (macrophages present); right-hand images, AP20187 (macrophages depleted). In both control and experimental mice marked luminal expansion is evident 1 day after removal of pups (some lumens outlined in black for illustration). In vehicle-only mice a marked decrease in lumen size by day three is observed as milk is resorbed. Very small lumens remain on day 7, however significant numbers of lipid filled adipocytes are evident between the alveoli starting on day three and increasing to day 7. These changes occur much more slowly in the glands of AP20197-treated mice, providing evidence that macrophages are required for several aspects of normal involution. *Source: Reproduced by permission from Ref. 183; link at Development:* dev.biologists.org.

proposed that clearance of shed (apoptotic) epithelial cells during the first 3 days after pup removal is accomplished by the remaining resident MECs by a process they refer to as efferocytosis from the Greek for "to take to the grave" or "to bury". Their elegant images depict apoptotic cells clearly being engulfed by epithelial cells in the monolayer.

However, in a subsequent study O'Brien et al.[183] clearly showed involvement of M2 macrophages in several aspects of involution, including clearance of apoptotic epithelial cells during the first 3 days following pup withdrawal. Further, in the absence of M2 macrophages involution was dramatically delayed and the rate of milk clearance was drastically reduced (Figure 46.9). Macrophages bearing markers of M2 development were clearly present and stained with anti-F4/80 antibodies in normal mice. When mice sensitive to a treatment that depletes macrophages were used, all aspects of involution were drastically slowed, and milk was not cleared from the luminal space 3 days after pup withdrawal. The sum of the evidence leads to the conclusion that both MECs and macrophages are involved in clearance of milk and shed cells.

An alternative hypothesis was put forth by Watson and her colleagues who suggested that a nonapoptotic form of programmed cell death was activated during involution; they proposed that lysosome-mediated cell death occurs during involution.[189] This process would occur independently of caspases 3, 6, and 7; however, it requires STAT3, which upregulates the expression of cathepsin B and L. Cathepsins B and L are dramatically activated during the first 3 days of involution corresponding to the first phase of apoptosis. Current evidence suggests that activation of STAT3 alone may not be sufficient to induce programmed cell death during involution since loss of M2-differentiated macrophages prevented this type of cell death even in the face of tyrosine-phosphorylated, activated STAT3.[183] Perhaps both activated STAT3 and M2 macrophages are necessary for involution to proceed, but neither alone is sufficient to support this process. It also remains possible that other processes are involved in this cell death, including autophagy, which appears to be involved in cell death in three-dimensional culture models of the mammary epithelium.[190,191]

Numerous molecules have been implicated in the apoptotic process. Some investigators have suggested that the relative balance between pro- and anti-apoptotic members of the BCL2 family of proteins is important in regulating apoptosis and involution.[192–195] Consistent with this hypothesis, overexpression of *Bcl2* delays involution,[193] as does overexpression of an activated form of AKT1, which is capable of phosphorylating and inactivating pro-apoptotic BCL2 proteins.[196] The relation of anti-apoptotic BCL2 family members to the formation of apoptotic mammary cells is currently unclear.

Milk stasis appears to be the major trigger for involution,[182] possibly involving increased mechanical stretch of alveolar epithelial cells due to expansion of the luminal space.[197] Two different groups have used global gene expression profiling to independently identify a group of genes whose expression changes during the first 12 h following pup withdrawal.[198,199] Both groups noted increased expression of genes encoding death receptors, and their ligands, proteins associated with the acute phase response, and most notably the transcription factor STAT3 and its targets.[198,199] Death receptor ligands included leukemia-inducing factor (LIF), tumor necrosis factor, tumor necrosis-like weak inducer of apoptosis, Fas Ligand, and TGFβ.[198,199] LIF activates STAT3, which in turns enhances expression of pro-death molecules; consistent with a critical role for STAT3 is the observation that cell death is delayed in STAT3-deficient mice and that the first phase of involution is extended for at least 6 days in the forced involution model in these mice.[200–202] Other critical changes include decreased expression of both AKT1 and insulin-regulated substrate-1,[196,203,204] which would eliminate the ability of AKT1 to suppress apoptosis. In addition to STAT3, the nuclear factor kappa B-signaling pathway is also likely to be an important component of the immediate signaling response to pup withdrawal.[205,206] It has also been suggested that decreased expression of the plasma membrane Ca^{++}ATPase 2, which is important in transport of Ca^{++} into milk,[207] results in an increase in intracellular Ca^{++} thereby stimulating apoptosis within the cell by a direct mechanism.[208]

Another important regulator of involution that has received extensive study is TGFβ. Although all three isoforms of TGFβ are expressed during pregnancy, expression of all three is downregulated at parturition, and only low levels of TGFβ3 are detected in alveolar cells during lactation.[209–212] Expression of TGFβ3 increases dramatically during milk stasis and involution.[198,199,211,213] Studies with transplanted MECs from TGFβ3 null mice support a paracrine role for this growth factor in mammary involution.[211] Expression of TGFβ from the whey acidic protein (WAP) promoter, which turns on in late pregnancy, inhibited alveolar development,[214] and it appeared that the stem cell pool available for alveolar morphogenesis was reduced.[215] Moses and others found that targeting a dominant-negative TGFβ Type II receptor (MMTV-DNTGRII) to the mammary gland inhibited TGFβ signaling and resulted in alveolar hyperplasia and premature secretory activation in pregnancy, followed by lactation failure.[216–218] This result is expected if TGFβ is inhibiting both alveolar proliferation and secretory activation. Expression of a constitutively active subunit of the Type I receptor leads to apoptosis in pregnancy and decreased proliferation in a transgenic mouse model.[219] More recently, in studies in which TGFβ type

II receptor was deleted from the mammary epithelium using Cre recombinase driven by the WAP promoter, the first phase of involution occurred normally, but TGFβ appeared to be required for commitment to the second phase of involution.[220] MECs lacking TGFβ-RII continued to express markers typical of a cell from a lactating mouse even 7 to 10 days after induction of involution.[220] These data all suggest a critical role for TGFβ in suppression of ductal and alveolar proliferation, suppression of PRL-induced gene programs, and programmed cell death of MECs particularly during the second phase of involution.

In contrast to the first phase of involution, commitment to the second phase of involution is dependent upon factors such as TGFβ as described earlier, and can be halted by treatment with glucocorticoid[179]; it has been speculated that glucocorticoid may act through maintenance of tight junctions.[221] The activation of matrix metalloproteases (MMPs) is critical in the remodeling of the tissue that occurs in the second phase, and although MMPs are expressed during the first phase of involution, their activation is held in check by tissue inhibitors of metalloproteases (TIMPs).[222,223] Substrates for MMPs include collagen IV, chemokine, and E-cadherin, and loss of these proteins causes detachment of epithelial cells and death by anoikis (detachment-induced cell death). The importance of MMP activation in the second phase is demonstrated by the observation that involution proceeds more rapidly in TIMP-3-deficient mice.[224] Recently it has been shown that MFG epidermal growth factor 8 (MFGE8) is essential for the second phase of involution as it binds to phosphatidylserine on the plasma membrane of apoptotic cells and enhances their phagocytosis.[225] Gene expression profiling studies by Stein et al.[199] suggest that the increase in pro-inflammatory signals during phase 1 of involution is important in the infiltration of inflammatory cells during the second phase,[199] but these signals could actually be important in attracting macrophages to the gland in the first phase.

Further details of the two phases of involution are available in numerous excellent reviews.[226–228] Future research should show how all these somewhat disparate pathways work together and also provide further understanding of the aspects of the microenvironment of the involuting mammary gland that promote mammary tumorigenesis.

CELL–CELL INTERACTIONS IN THE MAMMARY EPITHELIUM

Epithelial cells are joined to their neighbors by four types of junctional complex.[6] Starting from the apical pole of the cell these are: (1) the tight junctions (*zonula occludens*), which regulate passage of molecules through the paracellular pathway; (2) the adherens junctions (*zonula adherens*), thought to provide structural stability to the epithelium as a whole; (3) desmosomes (*macula adherens*), specialized for cell-to-cell structural adhesion; and (4) gap junctions made up of hexamers of connexin proteins that permit passage of molecules between cells. We will summarize the role of these junctions in turn as they apply to alveolar development and lactation.

Mammary Tight Junctions and Their Regulation

Production of milk of a defined composition requires that the milk space be firmly isolated from the fluids of the interstitial space, such that milk composition is entirely determined by the secretory activity of the mammary epithelial cell. By injecting a marked compound that could not cross cell membranes into the milk space and determining its appearance in the blood stream, Linzell and Peaker[229,230] showed in the 1970s that this isolation is complete in the lactating goat. Specifically they measured the transfer of injected [^{14}C]-sucrose from the lumen into the blood stream and could detect no transfer of the tracer during lactation. When a similar tracer was injected into the blood, it did not appear in the milk. Further, potential differences of −20 to −35 mV were found between the blood and the milk in lactating goats[231] and mice.[232] All these findings provide very strong evidence that the paracellular pathway of the mammary epithelium is highly impermeable during lactation.

The same is not true of the mammary epithelium during pregnancy. [^{14}C]-sucrose has consistently been observed to move from the blood stream into the milk at this stage of development,[136,229,230] and no electrical potential difference can be measured between electrodes in the blood stream and milk space. Moreover, transfer of macromolecules labeled with fluorescent tracers across the epithelium, either from the lumen to the interstitial space or in the opposite direction,[136,233] has been observed. These experiments, illustrated in Figure 46.10, show that during pregnancy molecules as large as albumin and IgA permeate the junctional complexes[111]; such molecules do not pass through the paracellular pathway in the lactating gland. When such large plasma proteins are present in milk, they must be transferred across the epithelium of the lactating gland by transcytosis as described in the section Transcytosis (Pathway III). Taken together these findings from isotope tracer, blood-milk potential, and fluorescent tracer experiments indicate that the mammary tight junctions, particularly in the alveoli, are leaky during pregnancy and close around parturition to form a tight barrier that prevents paracellular movement of molecules across the mammary epithelium.

This transition has a profound effect on the composition of the mammary secretion. During pregnancy, the

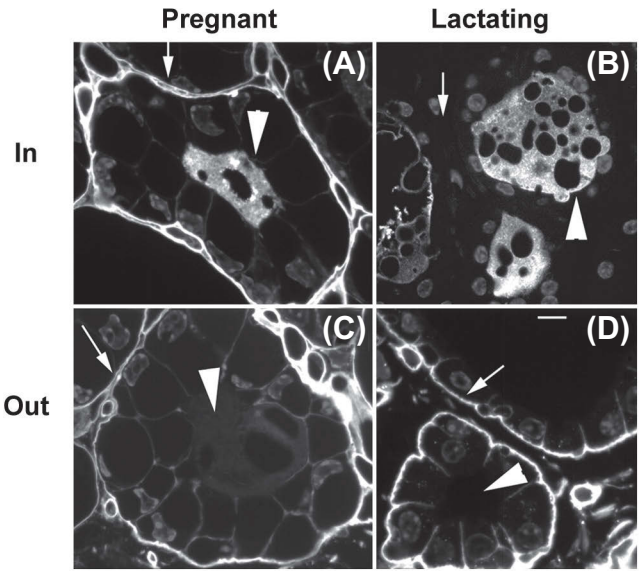

FIGURE 46.10 Paracellular permeability to FITC-albumin during pregnancy and lactation. (A, C) Pregnant mice. (B, D) Lactating mice. (A, B) FITC-albumin was injected intraductally (designated "In") and fixed within 5 min. The excised glands were embedded, sectioned, and visualized with the fluorescent microscope. During pregnancy, tracer can be seen in both lumen (arrowhead) and interstitial space (arrows); in lactation it is confined to the lumen. (C, D) The in situ gland was incubated with tracer for 1 h to expose the basolateral surface of the alveoli (designated "Out") and visualized as in (A) and (B). In pregnancy, tracer can be seen throughout the interstitial space and in the lumen; during lactation tracer is confined to the interstitial space extending just up to the tight junctions. Magnification bar (D) is 10 μm and applies to all panels. *Source: Reproduced from Ref. 136.*

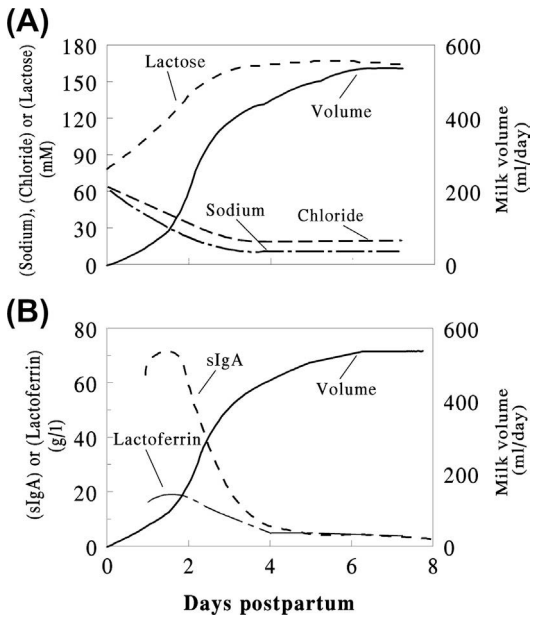

FIGURE 46.11 Changes in human milk composition during the colostrum-forming stage. (A) Na^+ and Cl^- concentrations fall rapidly and lactose increases as the tight junctions between the epithelial cells close. Note that these changes are well underway prior to the increase in milk volume secretion beginning on day 2. (B) Secretory IgA (sIgA) and lactoferrin are found at very high concentrations in the mammary secretion during the first three days postpartum, the period when colostrum formation is at its peak. *Source: Reproduced with permission from Ref. 160.*

milk has high Na^+ and Cl^- and low K^+ as the result of ions moving through the paracellular space down their electrochemical gradients. The low Na^+ and Cl^- and high K^+ of true milk is produced by the activity of ion pumps in the basolateral and apical membranes of the cells. The transition between the two states is well illustrated for human milk in Figure 46.11. Thus Na^+ and Cl^- fall and lactose rises (Figure 46.11(A)) as the tight junctions close prior to the increase in milk volume illustrated in both panels of Figure 46.11. Freeze fracture studies carried out by Pitelka[6] showed that the tight junction strands in the gland during pregnancy are highly disordered, suggesting that molecules permeate between the strands. In lactation the strands are highly ordered and show no breaks that might allow passage of large or small molecules.

Role of Claudins, Occludin and Tricellulin in Mammary Tight Junctions

Although claudins in low resistance epithelia permit the passage of monovalent ions,[234,235] the high transepithelial potential maintained in the lactating mammary gland despite substantial transcellular permeability to these ions suggests that the complement of claudins prevents paracellular movement of both large and small molecules. Our unpublished studies show a large increase in claudin 8 gene expression at parturition in the mouse mammary gland from both microarray and real time PCR analysis (Rudolph MC and Neville MC, unpublished). This claudin does not permit ion flux between lumen and interstitial space in other epithelia. Recently claudin 3 was localized to the apical junctions in the lactating mammary gland,[236] and the authors suggested that it is responsible for the low permeability of the mammary epithelium.

It is worth mentioning that claudins are also present in large numbers of small vesicles that appear never to interact with junctional complexes in the cell. Claudin 7 has been carefully studied in this regard,[83] but claudins 3 and 4 are also found in such vesicles in both normal and tumorous mammary cells in locations that suggest they are not merely serving as reservoirs for tight junction proteins (Baumgartner Wilson, personal communication). The function of these very common nonjunctional claudins is currently unknown, but they may play a role in interactions with the extracellular matrix.[237] Occludin is also present at mammary tight junctions and is considered to play a role in epithelial permeability; recent studies also implicate occludin as a signaling molecule that helps initiate apoptosis when tight junction integrity is compromised.[238]

The structure of the tight junctions at positions where three cells come together in the formation of the epithelial sheet appears to differ from that of the bicellular junctional complexes. Here a novel tetraspanin protein, tricellulin, is concentrated at the vertically oriented TJ strands of tricellular contacts. RNA interference studies of tricellulin depletion suggest that this protein is necessary for complete sealing of the epithelial barrier.[239] Recently another protein has been identified as a component of the tricellular junction, the lipolysis-stimulated lipoprotein receptor. This immunoglobulin receptor-like protein was actually found to recruit tricellulin to tricellular junction and again appeared necessary for full tight junction sealing.[240] In the liver it is involved in lipoprotein metabolism. Whether it serves this function in the mammary gland remains for future research.

Hormone Dependence of the Change in Tight Junction Permeability between Pregnancy and Lactation

When mice were ovariectomized on day 17 of pregnancy, the subsequent decrease in circulating P4 set the transition between pregnancy and lactation in motion, and the sucrose permeability of the epithelium progressively decreased over the 20 h subsequent to ovariectomy[136]; the effect could be delayed by injection of P4 at intervals. Injection of a P4 antagonist, RU486, produced the same effect as ovariectomy. These experiments firmly establish that P4 withdrawal is the trigger for tight junction closure. A series of endocrine ablation experiments showed that both PRL and glucocorticoid are necessary for mammary tight junction closure[241]; these molecules also alter tight junction permeability in tissue culture models.[242] These are the hormones that promote secretory activation in the mammary gland, providing evidence that tight junction closure is closely linked to secretory activation. Although insulin also promotes milk secretion in mice,[75,76] its role in tight junction closure is unknown.

Epithelial Permeability during Mastitis and Involution

The direct passage of interstitial molecules such as Na^+ and Cl^- across the mammary epithelium during pregnancy leads to the higher concentration of these ions in colostrum and the appearance of milk components such as lactose and α-lactalbumin in the blood stream as illustrated by the schematic in Figure 46.12. As discussed earlier, such changes in milk composition have been fully documented during the onset of lactation but have also been used as an indicator of tight junction status under conditions where direct tracer measurement of the permeability of the mammary epithelium is not possible such as during involution[243–245] and with infections such as mastitis.[246,247] Stelwagen and colleagues showed that increased milk components such as lactose and α-lactalbumin were found in the blood stream if goats and cows were left unmilked for 18 h.[245,248] The authors interpreted these results as showing an effect of milk stasis on tight junction permeability. However, in none of these studies were tracers injected into the gland or blood stream to distinguish between opening of tight junctions and aberrant basally directed exocytosis in the stretched mammary epithelium. Thus it is not clear whether the changes in ion concentrations in the milk result from a return of the tight junction strands to the disordered condition of pregnancy, a loss of integrity of the epithelium as epithelial cells are lost by apoptosis, or

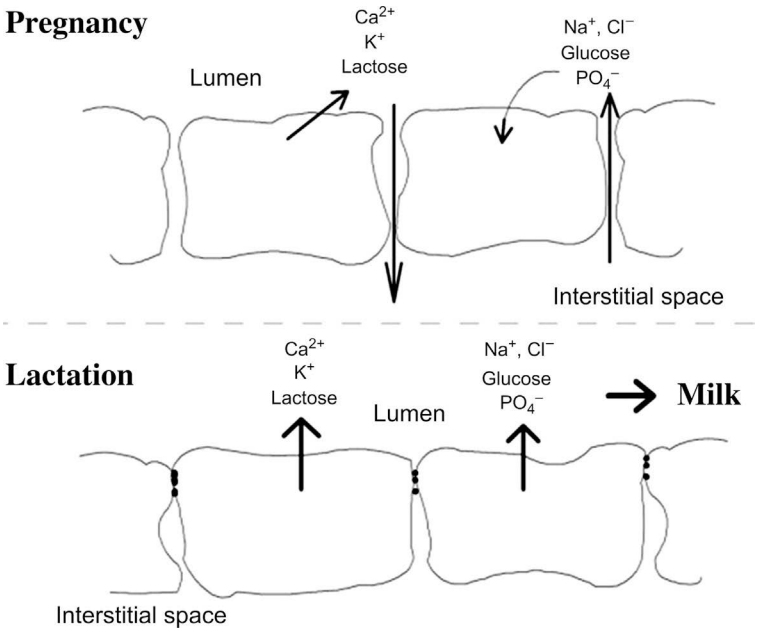

FIGURE 46.12 The paracellular pathway is open in pregnancy and closed in lactation. The schematic illustrates the net flux of several small molecules during pregnancy when the junctional complexes are very leaky and in lactation when they are tightly closed. As shown in Figure 46.10 large molecules such as albumin and α-lactalbumin are able to pass through the junctions during pregnancy. *Source: From Neville MC. Lactogenesis in women: evidence for a cascade of cellular events. In: Jensen RG, editor.* Handbook of composition of milks. *1st ed. San Diego: Academic Press; 1995. p. 87–98. Used with permission from Elsevier.*

activation of purinergic receptors in the mammary epithelium by mechanical or other disruptive stimulation.

Interestingly, it has been shown that cultured MECs release ATP, UTP, and UDP when stressed; these nucleotides interact with purinergic receptors to bring about an increase in cell Ca^{++} and activate Ca^{++}-sensitive apical Cl^- transporters[249] as well as alter Na^+ and K^+ transport.[250] It is possible that closure of junctions at the initiation of lactation is an irreversible process and that changes in both directional secretion of ions and milk components across the mammary epithelium may be responsible for alterations both in milk composition and the appearance of milk components in the blood when the gland is stressed by milk stasis or infection. The matter clearly requires additional investigation.

Regulation of Tight Junction Formation

Biochemically, tight junctions are complex structures, with claudins, occludin, and the junctional adhesion complex interacting across the intercellular space to form a tight apical band that restricts permeability through this space.[251] Intracellularly these molecules interact with scaffolding molecules immediately below the membrane called ZO-1, ZO-2, and ZO-3, which in turn interact with actin as well as members of the polarity-regulating complex PAR6/PAR3/aPKC.[252] A variety of regulatory molecules including PKCzeta[253] and the molecular complex CRUMBS3/PALS1/PATJ[252] have been shown to interact with the PAR complex and alter tight junction permeability.[254] A complete listing of tight junction proteins with their functional context is available,[255] with the suggestion that many of them may have a regulatory function in mammary development. Recently it has been shown that cytoplasmic polyadenylation element binding protein is necessary for claudin 3 and ZO-1 localization to both MECs in the mouse and in cell culture models of mammary acini.[256] Horseman and his group have implicated serotonergic signaling in tight junction integrity,[165] and work by Fischer et al. implicates the RHO pathway as well.[242] Occludin, a component of the tight junction, also plays a role in initiating apoptosis of MECs when tight junctions are perturbed.[238] At this writing a comprehensive picture of the interactions of all these signaling pathways in establishing and maintaining tight junction integrity in the lactating mammary gland is not yet available.

Adherens Junctions in the Mammary Epithelium

Adherens junctions encircle epithelial cells, usually basal to the tight junctions, and provide both cell–cell adhesion and linkage to the cytoskeleton through a group of molecules, the catenins. A transmembrane molecule, E-cadherin that interacts with the catenins, forms a Ca^{++}-dependent bridge from one cell to its neighbor, stabilizing cell–cell interactions. Disruption of these linkages by infusion of EGTA intraductally into the lactating mammary gland both decreases milk production and leads to apoptosis of MECs.[257] E-cadherin is essential for the self-organization of epithelial monolayers in culture models of the mammary epithelium[258] and was found to be necessary for establishment of the lactating phenotype in a Cre/loxP model directed to the mammary epithelium.[259] One of the catenin molecules, β-catenin, acts not only to stabilize the adherens junction but it is also a transcription factor, acting through the TCF/LEF pathway to regulate transcription and tumorigenesis by mechanisms well reviewed by Carraway and colleagues.[260]

Beta-catenin also functions as a transcriptional regulator in the WNT-signaling pathway[261]; its normal function was found to be essential for lobular-alveolar development.[261] Overexpression of the cytoplasmic domain of E-cadherin promoted precocious mammary development in mice but interfered with development of polarity in the fully differentiated gland.[262] Because loss of cadherin is considered to be a hallmark of the epithelial-to-mesenchymal transition that occurs during tumorigenesis,[263] much of the work on mammary adherens junctions is part of the breast cancer literature, which will not be reviewed here.

Desmosomes

Desmosomes are localized spot adhesions on the lateral sides of cells that use desmoglein and desmocolin to bind across the intercellular space possibly to prevent shearing forces from disrupting the epithelial monolayer. These structures have been found to be essential for branching morphogenesis of the mammary gland[264] and for formation of acinar cultures from purified MECs.[265] However, Pitelka and her colleagues showed almost 40 years ago that as the alveoli become secretory the desmosomes disappear,[6] likely to allow the profound shape changes in the epithelial cells as the lumens expand and contract with the accumulation and ejection of milk.

Gap Junctions in the Mammary Epithelium

Direct cell-to-cell signaling is mediated in part by the passage of molecules with molecular weights less than 1000 kDa through gap junctions. These junctions are formed from six connexin molecules that aggregate to form a hemichannel or connexon in each cell; when these hemichannels in the opposing membranes of two cells are aligned, they form a channel with a

pore that allows intercellular passage of signaling molecules, metabolites, vitamins, and other substances up to 1.5 kDa,[266] but not macromolecules. Freeze fracture studies showed that gap junctions between epithelial cells in the mammary gland are composed of an aggregate of many connexons.[267] Studies using lucifer yellow dye showed extensive coupling between cells of the lactating mammary alveolus as well as suggested dye transfer between alveolar and myoepithelial cells.[232]

Four connexins have been shown to be expressed in rodent mammary glands.[268] The mRNAs for connexin 26 (*Cx26*) and connexin 32 (*Cx32*) are expressed at all stages of mammary development[268,269] but are markedly upregulated in pregnancy (*Cx26*[269–271]) and lactation (*Cx26* and *Cx32*[271,272]). The corresponding proteins are found in the junctional plaques where they form both homomeric and heteromeric channels.[273] Connexin 30 (CX30) was expressed after day 15 of pregnancy and peaked at the onset of lactation, disappearing thereafter.[268,273] Gusterson and his colleagues speculate that the heteromeric CX26/CX30 channels specify cell–cell communication at late pregnancy and are replaced by heteromeric CX26/CX32 channels with differing specificity during lactation.[273] Connexin 43 (CX43) is the only isoform that has been positively identified in the myoepithelium.[274] It switches to a hypophosphorylated form during lactation, a change that may by specified by a loss of WNT-5A signaling.[275] Serra and her colleagues found that overexpression of WNT-5A in the mammary epithelium altered CX43 phosphorylation and led to impaired lactation with no change in any other junctional complex proteins. Based on this and other evidence, the authors suggested that CX43 promotes communication between myoepithelial cells essential for milk ejection.[275]

Talhouk and his colleagues have presented convincing evidence that gap junction formation is essential to the differentiation of MECs.[268] Although part of this effect may be coordination of epithelial cell activities between members of the epithelial layer including both basal and luminal cells, there is considerable evidence that gap junction proteins interact with regulatory proteins more generally thought to be associated with the tight and adherens junctions. As shown in Figure 46.13, the proteins for which there is current evidence are α-catenin, β-catenin, and ZO-2.[266] The authors propose that as they form, maturing gap junctions recruit β-catenin away from the nucleus, promoting a switch from proliferation to differentiation. Since gap junctions are lost in breast cancer cells, it is also possible that functional gap junctions help prevent transformation of MECs into tumor precursors.[266] This is a story worth following into the future.

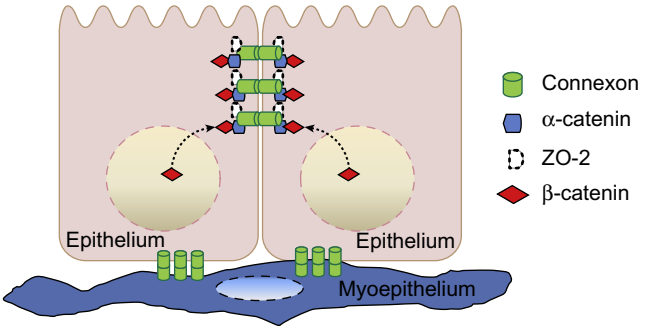

FIGURE 46.13 Gap junction localization in the differentiated mammary epithelium. Connexons interact as shown across the interstitial space between luminal epithelial cells as well as between luminal cells and myoepithelial cells. Luminal cell connexons also interact with signaling molecules α-catenin, β-catenin, and ZO2. As differentiation progresses, β-catenin may be recruited away from the nucleus to diminish the stimuli for proliferation. *Source: Reproduced with permission from Ref. 266.*

MILK COMPOSITION AND ITS REGULATION

Milk is qualitatively and quantitatively complex, containing proteins (mostly caseins), lipids (triglycerides), sugars (including oligosaccharides), vitamins, minerals, and growth factors, in addition to water.[169–171,276] The relative amounts of these substances vary significantly among species,[169,171] and the composition of milk can be influenced by the stage of lactation[28] and the mother's nutritional status.[277,278] Such variability implies that the mechanisms responsible for synthesis and/or secretion of milk substances are genetically determined and physiologically regulated. In this section we begin by summarizing the major milk components followed by a discussion of the major pathways for secretion of milk components and their regulation.

Major Milk Components

Genome-wide comparisons of mammalian taxa, including humans,[279] have led to greater understanding of how milk secretion evolved in mammals. These data suggest that the mechanisms of milk secretion developed over 160 million years ago and are highly conserved. Nevertheless, significant interspecies variability in genes encoding milk proteins as well as proteins involved in milk protein production have been detected. Such findings suggest that species variation in milk composition is likely to reflect differences in gene copy number and/or transcriptional or posttranscriptional mechanisms rather than major gene sequence differences.

Proteins

The protein content of human milk, initially about 3% (wt/vol), decreases to about 1.5% by the second week of lactation.[28] Caseins, which represent about 80% of total milk proteins,[280] form insoluble micelles containing high concentrations of calcium and phosphate. Other milk proteins are found in the soluble (whey) fraction, or are associated with the membrane that surrounds fat globules. A total of 285 distinct gene products have been identified in human milk; by comparing proteome data sets from human and bovine milk, a core of 106 conserved proteins has been identified.[280] Gene ontology analysis suggests that core mammary cell proteins fall into four general functional categories: cell proliferation, lipid metabolism, nutrient transport, and immune function.[280] Proteins enter milk through four distinct pathways as discussed in the section Milk Secretion Pathways, below.

Lactose and Oligosaccharides

Human milk is enriched in lactose (a disaccharide unique to milk) and oligosaccharides, relative to milk from most other species.[279,281,282] Total oligosaccharide concentration in human milk is higher in the colostral phase of lactation compared to that of mature milk. More than 200 different free oligosaccharides have been identified in human milk,[283] suggesting the presence of diverse mechanisms for oligosaccharide biosynthesis within human MECs. Analysis of oligosaccharides in the milk of other primates further suggests that primate milk oligosaccharides are generally more complex, and exhibit greater diversity, than those found in nonprimate milk.[284]

Lipids

Lipids in milk are primarily triglycerides (>98%),[285] which due to their high energy content provide the majority of the calories required for neonatal growth in most species.[286] Milk lipids also serve as a primary source of essential fatty acids needed for neonatal membrane synthesis, as substrates for synthesis of eicosanoids and other bioactive lipid signaling molecules, and they provide a mechanism for transfer of fat-soluble vitamins to infants.[276] The lipid content of milk is variable, with content differing among species and influenced by lactation stage.[287] In humans, the amount of lipid in mature milk is greater than that in colostrum, and it is positively affected by the degree of breast emptying.[288] It is thought that the total lipid content of milk is not significantly affected by diet for adequately nourished mothers.[77] However, more recent evidence from laboratory animals indicating that diet may influence milk consumption, and that maternal obesity may reduce milk lipid levels,[278] raises questions about possible negative influences of maternal overnutrition on human milk lipid content and offspring obesity risk.

Milk Secretion Pathways

Five general pathways are responsible for secreting the majority of milk products (Figure 46.14). Proteins, oligosaccharides, and some small molecules synthesized by MECs, as well as water are secreted by exocytosis (pathway I). Lipids, lipid-associated proteins, and membrane proteins are secreted by a unique membrane-envelopment process (pathway II). Externally derived macromolecular substances, including albumin, immunoglobulins, growth factors, cytokines, lipoproteins, and micronutrients enter milk by two pathways: transcytosis

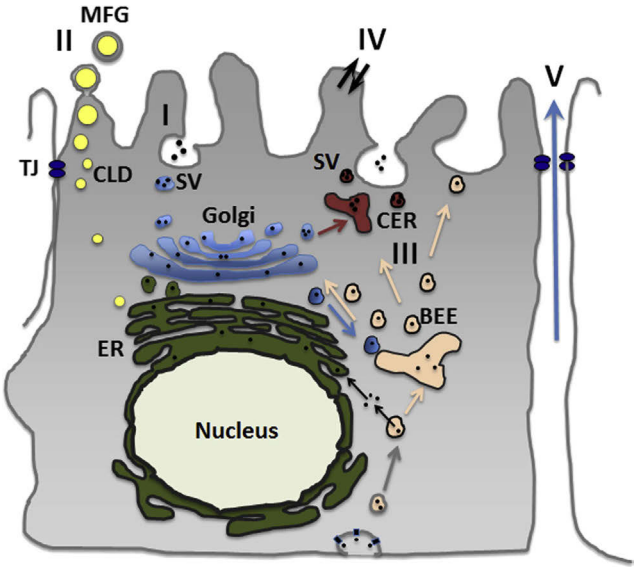

FIGURE 46.14 Cellular pathways for the secretion of milk. Five distinct pathways are responsible for the secretion of milk components. Major milk proteins, such as casein, and oligosaccharides, lactose, and water are packaged for secretion by exocytosis of secretory vesicles (pathway I) by processes originating in the Golgi complex. Lipids are synthesized and packaged into cytoplasmic lipid droplets (CLD) by enzymes in the endoplasmic reticulum. CLD are transported to the apical plasma membrane, where they are secreted by an apocrine process (pathway II) forming membrane-enveloped structures called milk fat globules (MFG). Immunoglobulins, and other macromolecules from the maternal circulation, are transported into milk by the transcytosis pathway (pathway III). In this pathway, substances taken up by either clathrin-dependent or clathrin-independent endocytosis at the basal plasma membrane initially enter into a basolateral early endosome (BEE) compartment where they are sorted to the trans-Golgi network for packaging into the secretory vesicles or to a common endosome recycling compartment (CER) for further sorting to apical or basolateral membranes. Direct movement of monovalent ions, water, and glucose across the apical and basal membranes of the cell occurs via membrane transporters (pathway IV). A paracellular pathway between epithelial cells, open during pregnancy, allows flux of plasma components into milk (pathway V). Tight junctions (TJ) close at the onset of lactation. *Source: From Monks J, Manaman JL. Secretion and fluid transport mechanisms in the mammary gland. In: Zibadi S, Watson RR, Preedy VR, editors.* Handbook of dietary and nutritional aspects of human breast milk. *Wageningen Academic Publishers, in press. Used with permission of J.L. McManaman.*

(pathway III), which involves elements of endocytic recycling and exocytotic pathways; and paracellular transport between cells (pathway V) prior to tight junction closure. Ions and small molecules, such as glucose and amino acids, are transported into milk by specific membrane transport pathways (pathway IV). Each of these pathways is affected by the functional state of the mammary gland, and is directly or indirectly regulated by actions of hormones and growth factors. Information exists about the general features of these pathways, however, few details are available about their mechanisms, or how their activities are regulated. The properties and regulation of pathway V are discussed in the section Cell–Cell Interactions in the Mammary Epithelium.

Exocytotosis (Pathway I)

During the secretory activation phase of mammary development, there is significant expansion of the rough endoplasmic reticulum and the Golgi complex, the organelles responsible for synthesis and packaging of proteins, lactose, and oligosaccharides into secretory vesicles for secretion into milk.[289–291] In species where it has been studied, the Golgi complex accounts for between 5% and 15%, and the rough endoplasmic reticulum accounts for approximately 25%, of the total volume of milk secreting cells at mid-lactation.[292] In addition to protein and oligosaccharide cargo, the Golgi complex packages nutrients, such as lactose and citrate, into secretory vesicles. The basic mechanisms by which secretory vesicle cargo is released during exocytosis were established over 40 years ago in studies of the pancreas.[293] The same mechanisms are thought to apply to essentially all exocrine cells, including those that secrete milk.

The Golgi complex is composed of seven cisternae that are classified as cis-, medial-, or trans-compartments based on their structural and functional characteristics.[294] Newly synthesized proteins are transported from the endoplasmic reticulum to the Golgi in vesicles that dock at the cis-compartment, where they are incorporated into new cisternae. The protein cargo remains within the cisternal lumen of the Golgi as cisternae progress to trans-portions of the complex.[295] Here they are sorted into transport vesicles and exit the Golgi complex. High-resolution 3D-electron micrograph tomography, in combination with rapid freezing procedures that stop cellular processes without disrupting cellular structural integrity,[296–299] has demonstrated that the trans-Golgi compartment is structurally and functionally distinct from other compartments. Vesicles destined for the endosomes or lysosomes are derived exclusively from the trans-most cisternae,[294,298] whereas vesicles destined for the apical and basolateral regions of the plasma membrane are derived from preceding cisternae.[294] Tomographic 3D reconstructions of Golgi in actively secreting cells in tissue culture[296,300] also demonstrate that direct tubule connections can form between cisternae. Such connections may facilitate cargo transfer between individual cisternal elements of the Golgi and promote secretory vesicle formation. Similar structural changes, if they occur among Golgi cisternae in milk-secreting cells, provide a mechanism by which the rate of secretion adapts to changing lactational demands of nursing young.

Exocytotic secretion requires fusion of secretory vesicle membranes with the plasma membrane to achieve cargo release. Fusion occurs at specialized sites on the plasma membrane called porosomes[301] and depends on the formation of a fusion complex between *N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF), soluble NSF-attachment proteins (SNAPs), and specific soluble NSF-attachment protein receptors (SNAREs) that are found on vesicle membranes and the plasma membrane.[302] Interactions among these proteins lead to fusion of vesicles with the plasma membrane, resulting in the release of the vesicle contents into the luminal space by an ATP-dependent process.[303] This general mechanism of exocytosis appears to be conserved among cell types,[304] and striking similarities exist in protein composition of the machinery that mediates regulated- and constitutive-exocytotic processes.[304] Members of the fusion complex are expressed in mammary glands of lactating mice,[305] and proteomic analysis of isolated Golgi from MECs has demonstrated that lactation leads to the upregulation of exocytotic machinery and vesicle trafficking proteins.[306] Localization studies have implicated SNAP-23, Syntaxin-2, and VAMP (vesicle associated membrane protein)-8 as possible mediators of casein secretion. However, the specific functional importance of these proteins to milk secretion processes has not been validated by gain or loss of function studies.

PROTEINS

Proteins are secreted by both constitutive and regulated pathways. The primary function of the constitutive pathway is the delivery of new membrane proteins, however in some cases it functions to deliver proteins for secretion.[307] Conversely, the regulated pathway specifically targets proteins to the plasma membrane for secretion in response to physiological signals. This pathway is the primary mechanism by which proteins are secreted in the pancreas, salivary, and adrenal glands. Elements of regulated secretory pathway have been identified by proteomic analysis of Golgi preparations from lactating rat mammary glands,[306] and evidence of regulated secretion of casein has been detected in isolated milk secreting cells.[308] However, this mechanism appears to account for only a small percentage of total casein secretion.

LACTOSE AND OLIGOSACCHARIDES

Lactose is synthesized within the Golgi complex by the transfer of galactose from UDP-galactose to glucose

in a reaction that is catalyzed by a complex between β-4-galactosyltransferase-1 and alpha-lactalbumin.[309] Biochemical studies showing that lactose is enriched in preparations of casein-containing secretory vesicles from mammary glands of lactating rats suggest that it may be secreted along with milk proteins.[310] Milk oligosaccharides are synthesized by addition of glycosyl groups, including *N*-acetylglucosamine, fucose, and *N*-acetylneuraminic acid to the galactose residue of lactose. This basic mechanism accounts for the synthesis of both linear and branched-chain oligosaccharides.[311] Relatively few details are known about the regulation of milk oligosaccharide synthesis, what determines oligosaccharide structural diversity, or how oligosaccharides are packaged into secretory vesicles for secretion during lactation. The various glycosyltransferase reactions responsible for generating specific oligosaccharide structures localize to different Golgi compartments,[312,313] suggesting the existence of specific mechanisms for detecting and regulating oligosaccharide abundance, as well as for transporting oligosaccharides within the Golgi to achieve specific structural characteristics. Specific domains within the C-terminal regions of glycosyltransferases control their localization within Golgi cisternae,[314] however, the mechanisms controlling glycosyltransferase targeting to a particular Golgi compartment have not been identified.

CALCIUM, PHOSPHATE AND CITRATE

Milk is rich in calcium, $PO_4^=$, and citrate, which exist in a variety of chemical forms including free ions, as Ca^{++} $PO_4^=$ or Ca^{++}citrate complexes, or bound to casein for Ca^{++} and $PO_4^=$ ions. Citrate is synthesized de novo by MECs, whereas milk Ca^{++} and $PO_4^=$ are derived from the maternal circulation. Biochemical, physiological, and kinetic evidence indicates that all three molecules are secreted into milk by exocytosis of Golgi-derived secretory vesicles.[315] Golgi membranes contain Ca^{++} pumps and citrate transporter activities that presumably mediate the transport of these substances into Golgi-derived vesicles.[316] Although it is likely that $PO_4^=$ enters the Golgi system by transport, in some species Golgi $PO_4^=$ may also originate by UDP hydrolysis during lactose synthesis. Although some of the Ca^{++} in milk must be transported from the exocytotic pathway, clear evidence for a powerful apical membrane transporter, PCMA2, that transports the majority of Ca^{++} in rodent and bovine milk, has been obtained from several laboratories.[207,317,318]

Lipid Secretion (Pathway II)

The mechanism of milk lipid secretion is distinct from the exocytic pathway used to secrete proteins, sugars, and water into milk and from exocytic pathways used by hepatocytes or enterocytes to secrete lipids into the circulatory system.[319] Milk lipids originate from CLD,[320–322] which are secreted into milk as membrane-bilayer coated structures, known as MFG, through an apocrine mechanism.[322,323] Proteomic analyses,[324] which documented that the overall protein composition of CLD isolated from milk secreting cells is similar to that of the secreted MLG, suggest that CLD are secreted into milk in toto, without significant modification of their compositions. Such studies also demonstrated that the protein signature of CLD isolated from milk-secreting cells is distinct from that of CLD isolated from hepatocytes, which are not secreted, suggesting that protein composition of mammary CLD may be specialized for transport and secretion. The reason why milk-secreting cells have evolved a specialized mechanism(s) of lipid secretion that bypasses the endoplasmic reticulum and Golgi packaging machinery used in the secretion of other substances is unclear. However, the presence of a "direct lipid secretion" pathway may provide means of delivering large amounts of lipid to neonates during critical growth periods.[319] In addition, the membrane surrounding MFG protects milk lipids from emulsification and may possess additional biological functions.[325–327]

Based on ultrastructural evidence showing CLD contacting and being surrounded by the apical plasma membrane,[320] it has been proposed that CLD are secreted by a "membrane envelopment" mechanism.[322] Although most evidence favors this mechanism,[322,328] an alternative mechanism involving interactions between secretory vesicles, CLD, and the plasma membrane has been proposed.[329] In support of this alternative pathway, the SNARE protein SNAP-23, which has been implicated in the secretion of casein vesicles, has also been detected on CLD by immunofluorescence microscopy.[305] The possibility that CLD may be secreted by more than one mechanism is further suggested by observations that lipid-containing structures with a diverse range of sizes and lipid compositions have been detected in milk.[330]

Details about the mechanisms by which CLD form contacts with the apical plasma are limited. However, CLD are known to accumulate near the apical plasma during lactation,[289] and ultrastructural analyses have shown that they are separated from the plasma membrane by a 10–20-nm-thick layer of electron dense material.[290] Material of similar appearance has been shown to separate the surface of the lipid droplet from the surrounding membrane in secreted MFG.[290,331] Proteomic analyses of human, bovine, caprine, and mouse MFG membranes have shown that they are uniquely enriched in three proteins: the apical plasma membrane protein butyrophilin (BTN); the cytoplasmic enzyme, xanthine oxidoreductase (XOR); and the CLD-associated protein, adipophilin (ADPH/ADRP/perilipin 2).[332] Biochemical and immunocytochemical analyses indicate that these proteins exist as a stable complex in MFG,[333] and loss of function studies suggest that each protein contributes to normal milk lipid secretion.[334–337]

How BTN, XOR, and ADPH interact to mediate lipid secretion is not well understood at the molecular level, but interactions between these proteins have been documented and critical functional domains are beginning to be identified. ADPH on the CLD surface is proposed to form a complex with BTN and XOR that allows CLD to dock at the apical plasma membrane.[332] This mechanism is supported by experiments showing that ADPH exists as a complex with BTN and XOR on MFG membranes and co-localizes with these proteins on the apical plasma membrane at sites of CLD secretion.[107] Binding studies have further shown that XOR binds tightly to a cytoplasmically oriented domain, B30.2, of mouse BTN.[338,339] The C-terminal region of ADPH, which forms a four-helix bundle structure,[337] has also been implicated in CLD secretion by experiments in which mutant ADPH lacking the four-helix bundle domain was shown to disrupt CLD secretion in lactating mice.[337] Four-helix bundle motifs bind to phospholipid membranes and have been implicated in the induction of membrane curvature[340,341] and the recruitment of other proteins to the curvature site.[341] It is an intriguing possibility that interactions between the C-terminal four-helix bundle domain of ADPH and the apical plasma membrane of milk-secreting cells may initiate changes that lead to BTN and XOR recruitment to CLD contact sites and ultimately to the envelopment and secretion of CLD.[342]

Transcytosis (Pathway III)

Transcytosis provides a pathway for proteins and other macromolecules from the maternal circulation to be transported into milk. Substances transported by this pathway are taken up either by clathrin-dependent or clathrin-independent endocytosis at the basal plasma membrane of milk secreting cells and enter into a basolateral early endosome (BEE) compartment. Substances within this compartment are sorted into a rapid recycling pathway, to late endosomes/lysosomes, to the trans-Golgi network, or to a common endosome recycling (CER) compartment.[343,344] There is further sorting of substances within the CER to the apical membrane or back to the basolateral membrane.[344] Thus, transcytosis involves a series of complex sorting events that may or may not intersect with the exocytotic pathway used for secreting endogenously synthesized proteins, lactose, and oligosaccharides. The extent to which the exocytotic and transcytosis pathways intersect in the secretion of milk substances derived by endocytosis is unknown. However the demonstration that concanavalin A-ferritin complexes taken by endocytosis were found in secretory vesicles containing casein[345] provides experimental evidence of endocytic and exocytotic pathway intersection in the milk secreting cells of lactating animals.

IMMUNOGLOBULINS

Of the substances transported into milk by transcytosis, immunoglobulin A transport (IgA) is the best understood. Immunoglobulin A exists as a dimer (dIgA) and is by far the predominant immunoglobulin class in human milk.[346] At the onset of lactation, plasma cells home to the mammary gland where they lodge in the interstitial spaces, producing much of the IgA secreted into milk. Dimeric IgA synthesized by plasma cells or elsewhere in the body binds to polymeric immunoglobulin receptors (pIgR) on the basolateral surface of milk-secreting cells.[347] The entire IgA–pIgR complex is internalized by clathrin-dependent endocytosis and transferred to the apical membrane. In other cell types, dIgA–pIgR transcytosis has been shown to involve the BEE and CER compartments and an apical recycling endosome compartment.[348] Fusion of endocytic vesicles with the apical plasma membrane delivers the dIgA–pIgR complex to the apical plasma membrane, where the receptor undergoes proteolytic cleavage at arginine 585 to release dIgA bound to a portion of the pIgR extracellular domain called secretory component.[347] The mechanism of fusion of dIgA–pIgR vesicles with the plasma membrane in kidney cells involves *N*-ethylmaleimide sensitive factor (NSF) and components of the SNARE machinery.[350,351] Presumably a similar mechanism operates in milk-secreting cells, but the process has not been studied. Transgenic pIgR expression in mouse mammary glands demonstrated that pIgR levels limit dIgA transport into milk.[351] Dimerization of pIgR, induced by dIgA binding, stimulates the transcytosis of the complex in cultured immune cells,[352] possibly by activating PKC/PI3 kinase signaling pathways.[344] It is unknown if similar processes regulate dIgA transfer into milk.

SERUM ALBUMIN

In addition to transporting dIgA, elements of the dIgA–pIgR pathway also appear to be responsible for transcytosis of serum albumin into milk.[233] Fluorescently labeled serum albumin is found in close proximity to dIgA on the basolateral membrane of milk-secreting cells of lactating mice, and it co-localizes with dIgA in endosomal vesicles within these cells.[233] However, the identity of the vesicular compartment containing dIgA and serum albumin was not defined, and it remains unclear if dIgA and serum albumin undergo co-transcytosis at each step of the pathway. Nevertheless, co-localization analyses of serum albumin and casein demonstrated that the albumin transcytosis pathway does not intersect with the post-Golgi compartment mediating milk protein secretion.[233] Receptor mediated uptake has been implicated in the transport of albumin across various epithelial barriers. The presence of approximately 30 times more serum albumin than dIgA in mouse milk suggests the existence of mechanisms for concentrating albumin, or the presence of albumin receptors, for

transport into milk that differ from those for dIgA. In contrast to mice, where the serum albumin concentration in milk is approximately equal to that in serum,[233] the serum albumin concentration in human milk is significantly lower than that in serum.[353] Thus there appear to be species differences in aspects of serum albumin transcytosis into milk.

TRACE ELEMENTS

Transport of trace elements, such as iron, copper, and zinc, combines elements of transcytotic and exocytic pathways. Distinct mechanisms exist for trace element uptake into milk-secreting cells. Like IgA and albumin, iron is internalized by a receptor-mediated endocytotic process. Iron bound to transferrin (TFN) binds to the transferrin receptor (TFNR) on the basolateral surface of the mammary gland. The TFN–TFNR complex is internalized by clathrin-dependent endocytosis and transported into the BEE of the mammary epithelium, where the acidic environment releases iron from TFN. It is thought that released "free" iron is transported out of endocytic vesicles into the cytoplasm by the divalent metal ion transporter identified in hepatic cells and placental tissue,[354,355] but direct evidence that this process occurs in milk-secreting cells is lacking. In the cytoplasm, iron complexes with iron-binding proteins, such as ferritin, or it is transported into the secretory machinery by ferroportin where it complexes with transferrin or lactoferrin and is packaged into secretory vesicles carrying milk proteins.[356] While significant amounts of iron are transported into rodent milk to meet the nutritional demands of the growing offspring, human milk iron is very low as the full-term infant stores enough iron from the breakdown of fetal hemoglobin to meet infant demand for four to six months postnatally.[357]

Copper is transferred from the circulatory system into milk-secreting cells by basolateral located transporters. Three copper transporters have been identified in the mammary gland: Copper transporter 1 (CTR1), ATPase7A (ATP7A), and ATPase7B (ATP7B). CTR1, an essential regulator of copper import in most tissues,[358] including the mammary gland,[359] is found at the plasma membrane and forms a multimeric complex having a central copper channel with high affinity for copper.[358] ATP7A and B are polytopic transmembrane cation-transporting P-type ATPases that are closely related to each other.[360] Both proteins are found in the secretory pathway and function in the transfer of copper to copper-binding proteins or copper-dependent enzymes in this compartment.[361] Copper complexed to ceruloplasmin or metalothionine is packaged into secretory vesicles and secreted by the exocytotic pathway. Mutations in ATP7A and B are responsible for copper excretion defects observed in livers of Menke and Wilson disease patients. In each case, the defects are associated with impaired trafficking of these proteins from the Golgi to secretory vesicles.[362] ATP7A and 7B have been implicated in the secretion of copper into milk.[356] However, only impaired ATP7B function has been shown to influence milk copper levels in mice.[363] Transgenic studies in fact suggest that ATP7A mediates copper secretion from the basolateral membrane of the mouse.[364] Although ATP7A and 7B are expressed in human MECs,[364,365] their roles in milk copper secretion are not fully established. Milk copper levels are reported to be lower in patients with Wilson's disease,[366] however Wilson's disease does not appear to interfere with lactation.[367] Whether ATP7A is able to compensate for ATP7B dysfunction in the milk copper secretion in Wilson's disease patients is unknown. However, ATP7B has been shown to rescue copper accumulation defects observed in cells derived from mice with defective ATP7A,[360] thus overlap in the functions ATP7A and B may account for the limited effect of ATP7B loss on mammary gland function and milk copper levels.

The organization of the zinc secretory system is broadly similar to the copper secretory system in that specific transporters mediate zinc uptake from the maternal circulation and its subsequent transport from the cytoplasm into the secretory system. Twenty-four different zinc transporters (ZnT) have been identified in mammals; they fall into two categories, 10 ZnT and 14 zinc import proteins (ZIP).[368] ZnT family members transport zinc from the cytoplasm across the plasma membrane or into the secretory or endosomal pathways.[369] ZIP family members mediate transfer of zinc into the cytoplasm from the circulation or intracellular compartments.[370] ZnT family members are known to be critical regulators of zinc transport into milk of humans and rodents. In humans, a missense mutation in ZnT2 has been identified that is associated with low milk zinc levels,[371] and in mice a spontaneous ZnT4 mutation appears to be responsible for decreased milk zinc levels associated with the lethal milk phenotype. Specific roles for ZIP family members in zinc secretion into milk have not been described.

Membrane Transport (Pathway IV)

Na^+, K^+, and Cl^- ions are the most prevalent minerals in most milks, comprising about 10% of the osmolality of human milk, 25% of the osmolality of bovine milk, and about 65% of the osmolality of rabbit milk,[372] with lactose making up most of the rest. Because the total concentrations of Na^+ and K^+ in milk are equivalent whether determined by ion selective electrodes or flame photometry,[373,374] these ions are considered to be free, e.g., not complexed with other molecules. The same assumption is usually made for Cl^-.[375] The important questions are: What are the concentrations of these ions within the various compartments of the mammary tissue? What are the mechanisms by which the

TABLE 46.1 Monovalent Ion Concentrations in the Guinea Pig Mammary Gland

		Cell, mEq/L		Milk, mEq/L	
Component	**Interstitial, mEq/L**	**Guinea Pig; Ruminant**	**Mouse**	**Guinea Pig; Ruminant**	**Mouse**
Sodium (Na^+)	150	41.7	47	8	27
Potassium (K^+)	4.5	122 (free); 143 (total)	129	24	47
Chloride (Cl^-)	116	66.5		12	N/A

Data from Ref. 5.

concentration gradients between interstitial fluid, cell, and milk are maintained in the lactating epithelium? The first question was answered at least for goats and guinea pigs in a 1971 publication by Linzell and Peaker.[5] Table 46.1 shows the concentrations of Na^+, K^+, and Cl^- in the interstitial space, the cell water, and the milk. Similar results were reported for goats, cows, and sheep.[5] Before dealing with potential mechanisms for monovalent ion transport, it is useful to reiterate the basic principles proposed by Linzell and Peaker in 1971:[5]

1. All mammary membranes are freely permeable to water so that the concentrations of osmolytes essentially determine the volume of milk.
2. The ducts have the same ionic permeability properties as the alveolar cells, and milk composition is unchanged as it passes through the mammary ducts.
3. The ionic concentration in the cytoplasm is maintained by pumps and exchangers present on the basolateral membranes of the cells.
4. The apical membrane is permeable to Na^+, K^+, and Cl^-.
5. Na^+ and K^+ are at electrochemical equilibrium across the apical membrane, whereas the concentration of Cl^- in the milk is lower than predicted by passive distribution.

These concepts have not changed since Linzell and Peaker first proposed them[5]; they apply during full lactation when the paracellular pathway is closed.

WATER PERMEABILITY OF MAMMARY TISSUES

The major evidence for the free permeability of the mammary membranes, both epithelial and endothelial, to water is that milk is iso-osmotic with plasma under any circumstances in which it has been measured.[376,377] An interesting example is that during Ramadan, when women fast, their blood becomes slightly hypertonic and their milk follows suit.[378] Water channels called aquaporins are thought to be responsible for the water permeability of cell membranes. Aquaporin 1 (AQP1) was clearly shown to be present in myoepithelial cells in the bovine mammary gland as well as the capillary endothelium.[379] Aquaporin 3 (AQP3) was found on the basolateral membranes of secretory epithelial and ductal cells in the rodent mammary gland[380] and more selectively in the bovine gland.[379] Aquaporin 5 (AQP5) was present on the apical membranes of the mammary ducts of virgin animals[381] and possibly intracellularly during lactation. Aquaporin 7(AQP7), a water and glycerol channel, has been shown to be expressed in the murine mammary gland at lactation (Ramanathan and Neville, unpublished) but its localization is unknown. It can be assumed that these channels are at least partly responsible for water permeation across mammary membranes, but final answers are not yet in.

ROLE OF THE DUCTAL EPITHELIUM IN THE CONCENTRATION OF IONS IN MILK

Although the concentration of many secretions such as saliva is altered as the fluid passes through the ducts, the mammary gland appears to be an exception. The best evidence is that the concentration of ions is the same in milk obtained early in milking, which presumably has lingered in the ducts, as in milk obtained at the end of milking as long as high levels of OT are not used to obtain milk letdown.[382]

BASOLATERAL MEMBRANE TRANSPORTERS

There is abundant evidence for a number of pumps and exchangers in the basolateral membrane. The functional and immunohistochemical evidence for a ouabain-sensitive Na/KATPase in the basolateral membranes of the lactating mammary alveolar cells was first reported in the early 1970s[229,383] and has since been obtained in many laboratories (reviewed in Ref. 376); it is widely accepted that the Na^+ and K^+ levels in the mammary cell are mostly maintained by this enzyme. However, Na^+ levels in the cell (43 mM in the guinea pig, for example, 26 mM in the mouse[384]) are higher than in many other cells. The mechanism is not clear, but many Na^+-dependent exchangers are thought to be present on the basolateral membrane,[376] which could account for excess entry of this ion into the cell (Figure 46.15). Evidence for an amiloride-sensitive Na^+/H^+ exchanger as well as a DIDS-sensitive Na/HCO_3^- cotransporter were obtained in the 31EG4 cell line,[385] a nontransformed mouse mammary cell line derived

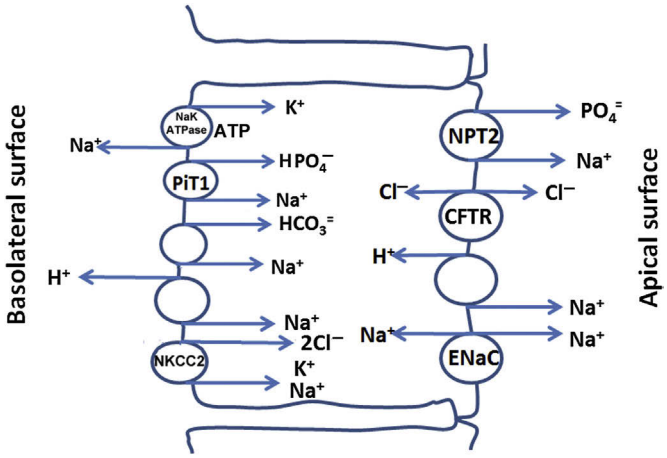

FIGURE 46.15 Transporters and channels in the basal and apical membranes of the mammary alveolar cell. This figure shows the membrane transporters for monovalent ions for which there is evidence from studies in the lactating mammary gland and tissue culture models. PiT-1 is the product of the Slc20a1 gene; the $Na^+/PO_4^=$ transporter Npt2, also known as NaPi-IIb, is the product of the Slc34a2b gene; the $Na^+K^+2Cl^-$ transporter NKCC2 is the product of the Slc12a2 gene; CFTR is the cystic fibrosis Cl transporter encoded by *Cftr* in the mouse: and ENaC is the nonvoltage sensitive amiloride sensitive Na^+ channel encoded by the murine Scnn1b gene. While Na^+ hydrogen exchangers have been proposed for both membranes, their molecular identity is not yet clear.

from the IM-2 cell line.[386] Shennan and colleagues[387] obtained physiologic evidence for electroneutral $Na^+/K^+/2Cl^-$ exchange in mammary explants inhibitable by furosemide. Clear immunohistochemical evidence supports the presence of this transporter at the basolateral membrane.[122,381,388] Levels were highest in the ducts of the virgin gland, but the protein was detected throughout pregnancy and lactation generally localized to basolateral membranes. Mice with a null mutation of this transporter showed no obvious defects in mammary morphology during lactation; however, the pups failed to thrive.[388] What could not be determined from the experiments was whether the lactation failure was due to a defect endogenous to the mammary gland or some other deficiency, since the mice have defects in a number of tissues.

APICAL MEMBRANE TRANSPORT

Apical membrane transport has also been characterized in a number of laboratories. The earliest experimental evidence was obtained in goats in 1974. Linzell and Peaker found that NaCl and KCl solutions infused up the teat were absorbed into the blood stream, whereas isotonic sucrose solutions infused in the same way drew ions into the milk space.[230] In addition, measurement of the effect of changes in the Na^+, K^+, and Cl^- concentration in the milk space of the goat mammary gland on the transepithelial potential difference provided evidence for the presence of a nonselective cation channel and a Cl^- channel in the apical membrane.[389] Additional evidence for K^+ and Cl^- channels comes from studies of ion transport across membrane vesicles isolated from MFG membranes.[390,391] In the guinea pig (Table 46.1), rat, and rabbit the ratio of the concentrations of Na^+ and K^+ in milk and mammary gland was approximately equal at 3, and both were at approximately electrochemical equilibrium across the apical membrane, suggesting that they are passively distributed.[372] This was also the case in the mouse where careful measurements of the transepithelial and basolateral potential in vivo, gave a transepithelial potential of −35 mV and a basolateral potential of −49 mV. By subtraction the apical potential should be in the range of −14 mV, not too different from the equilibrium potentials of Na^+ and K^+ across the apical membrane of −26 and −15 mV, respectively.[232,384]

The Cl^- concentration is much higher in the cell than in the milk of all species where it has been measured,[372] and the ion is obviously out of electrochemical equilibrium. The mechanism by which milk Cl^- is maintained at a low level is not clear, but experiments in tissue culture models are beginning to show how Cl^- transporters may function. The cell line, 31EG4, mentioned before, is a reasonably satisfactory model as it forms a tight monolayer when grown on a Transwell support. The resistance of this monolayer is substantially increased by glucocorticoid in a manner reminiscent of the in vivo mammary gland.[392] 31EG4 cells were found to possess both the amiloride sensitive epithelial Na^+ channel (ENaC) and the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel that is stimulated by cyclic AMP and inhibited by diphenylamine-2-carboxylate.[392] Both were localized to the apical border of 31EG4 cells by immunostaining, a location that was confirmed by electrophysiological experiments. CFTR was also identified in sections of human mammary gland at the apical border of the epithelium.[392] This transporter could be responsible for Cl^- movement between the milk and the cell in the lactating mammary gland: however, because it is a passive channel, it cannot account for the movement of Cl^- down its electrochemical gradient from the lumen into the cell. A further difficulty with an important role for CFTR in the ionic composition of milk is that women with cystic fibrosis, a disease with defective CFTR, have no difficulty with lactation.[393] While considerable physiologic evidence indicates the presence of a K^+ channel in the apical membrane,[376] the molecular identity of this channel remains unknown, unless it is the Ca^{++}-activated $K_{Ca}3.1$channel described following. There appears to be a good deal to be learned about the molecular nature of the channels in the apical membrane of the mammary epithelium.

PHOSPHATE TRANSPORT IN THE MAMMARY GLAND

Na^+ $PO_4^=$ cotransport in the mammary gland of the lactating rat was identified physiologically in 1996[394] when it was thought to mediate basal transport of both ions into the mammary cell in vivo. It is hypothesized that this transport is mediated by a Type III neutral Na^+ HPO_4^- transporter known as *PIT* encoded by Slc20 genes.[395] *Slc20a1* is upregulated at lactation in the mouse (Ramanathan and Neville, unpublished), suggesting that PIT-1 is the basal Na^+ HPO_4^- carrier in the mammary epithelium. A specific Na^+ $PO_4^=$ transporter (NPT2B or NaPi-IIB; gene name *Slc34a2*) was shown to be highly expressed at the apical membrane of the lactating mammary gland in the mouse[381] and goat[396] and can be either electrogenic, transporting Na^+ $PO_4^=$, or electroneutral, transporting Na^+ HPO_4^-.[395] These findings suggest that Na^+ and $PO_4^=$ are transported across the basolateral membrane into the cell by PIT-1 and across the apical membrane to the milk by NPT2B. However, detailed proof of this hypothesis is lacking.

A POTENTIAL ROLE FOR PURINERGIC RECEPTORS IN REGULATION OF MAMMARY GLAND ION TRANSPORT

Mammary tumor cells were found to secrete the nucleotides UTP and ADP after mechanical stimulation; these compounds in turn increased intracellular Ca^{++} in the tumor cells.[397] The receptor responsible for these nucleotide effects was found to be the metabotropic P2 purinergic receptor, P2Y. Blaug and colleagues[249] using 31EG4 cells showed that ATP and UTP stimulated apical Cl^- movement by acting on P2Y to increase Ca^{2+} release from the endoplasmic reticulum. Studies of purinergic receptor activation in primary human mammary epithelial cell cultures produced evidence for $K_{Ca}3.1$channels. K^+ intermediate/small conductance Ca^{++}-activated channel, subfamily N, member 4, also known as KCNN4, is a human gene encoding the $K_{Ca}3.1$ protein, which is responsive to P2Y receptors in the basolateral membrane of these MECs. Increased cellular Ca^{++} also activates ENaC in the basal membrane to alter Na^+ transport.[250] The physiological meaning of these findings is not entirely clear, but they indicate that Na^+ and K^+ secretion into milk is subject to a higher level of regulation than previously thought.

In conclusion, the major outlines of monovalent transport into milk have not changed since they were first proposed in 1971 by Linzell and Peaker.[5] However, considerably more molecular information about the mechanism of transport across the basolateral membrane is available. Many questions remain about the molecular mechanisms of apical transport.

MEETING THE ENERGY REQUIREMENTS OF LACTATION

The energy demands of lactation are significant and require a major shift in energy homeostasis. Some animals can meet this energy demand wholly by mobilizing endogenous energy stores.[398] Such animals (some bears, seals, baleen whales) usually have a large body size and the storage capacity that allows them to remain fasted throughout lactation. For most animals, however, the energy consumed in the diet is needed to support the demands of this expensive process. One adaptation of maternal physiology is the growth and increased absorptive surface area of the alimentary tract to extract the available nutrients from dietary intake.[399,400] This adaptation, in isolation, however, would be insufficient. To meet these demands, multiple signals converge to increase maternal caloric intake, minimize the energy requirements in nonessential tissues, and direct nutrients that will be used for milk production to the mammary gland.

Exogenous Nutrients

Much of the research on the role of exogenous nutrients in providing substrate for milk synthesis has been carried out in rodents, animals with large litters that require an enormous increase in energy transfer to support their high rate of growth. While the general adaptive mechanisms to meet these demands are thought to be present in most lactating animals, including humans, there are certainly species-specific adaptations. Here we will deal mainly with the extensive information available from studies in rodents as well as earlier studies in dairy animals.

Increasing Energy Intake

Two primary hormonal signals are thought to be involved in promoting hyperphagia during lactation: (1) PRL release associated with suckling; and (2) a decline in the adiposity-associated hormone, leptin (Figure 46.16).

Suckling can stimulate energy intake in the absence of milk production,[401–403] even when peripheral signals of negative energy imbalance are not present.[404] Suckling is a major stimulus to PRL secretion,[405] and PRL is thought to promote feeding in order to meet the high energy demands of lactation. PRL induces hyperphagia in nonlactating dams in a dose-dependent manner[406–408] and rescues the inhibitory effects of bromocriptine on the appetite of galactophore-cut dams (dams who are still being suckled but not producing milk).[409] This effect on appetite is thought to involve key areas of the hypothalamus that regulate energy balance. Both PRL and suckling can increase the expression of orexigenic (e.g., appetite promoting) neuropeptide Y in the dorsomedial hypothalamus.[410,411] In addition, these areas of the brain

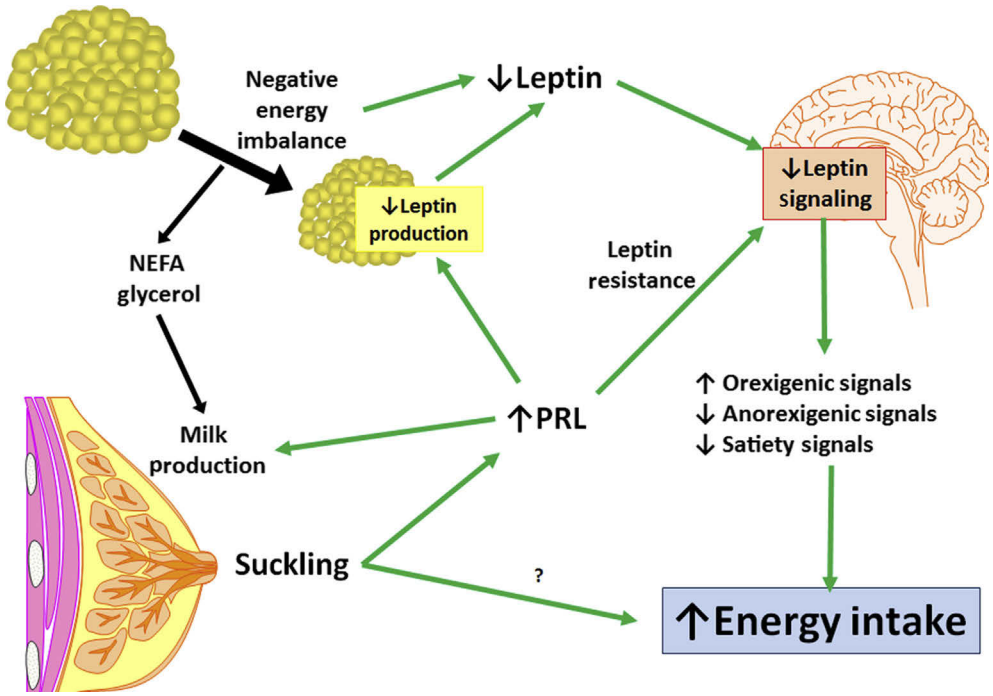

FIGURE 46.16 The regulation of energy intake during lactation. PRL secretion reduces the sensitivity of the brain to leptin. Suppressed leptin levels and central leptin resistance converge to promote feeding. Other signals from the suckling response may also contribute to this interplay between the mammary gland, adipose tissue, and the brain. The neural pathway linking suckling to energy intake is currently unclear as indicated by "?". The increased energy intake helps meet the energetic demands of lactation. PRL = prolactin; NEFA = nonesterified fatty acids.

become resistant to the anorexigenic (anti-appetite promoting) actions of leptin. PL signals through the PRL receptor and promotes hypothalamic leptin resistance by mid-pregnancy in the rat.[412,413] Central leptin resistance is maintained throughout lactation,[414] likely by PRL,[415] and appears to be essential for the maintenance of hyperphagia.[416–419]

Leptin signaling during lactation is also suppressed by decreased levels of the hormone.[403,420] Circulating levels become less responsive to meals and more responsive to maternal energy balance, while displaying a diminished diurnal fluctuation throughout the day.[403,420] The decline in leptin levels may result from the depletion of maternal adipose stores during lactation, as endogenous lipid stores are trafficked to the mammary gland.[403,404,418] Alternatively, PRL may directly inhibit the secretion of leptin.[421] Regardless of the mechanism, with a brain that is resistant to leptin, and leptin levels falling, the inhibition of orexigenic signals is diminished and the stimulation of anorexigenic signals is minimized, both of which result in increased appetite.[422]

Adaptations in Expended Energy

Lactation is an expensive process, particularly in rodents who increase their metabolic requirements up to threefold to nourish a rapidly growing litter of pups. This increased requirement is founded not only on the energetic value of the milk constituents but also in the maternal energy expended to produce and secrete the milk. In women, the efficiency of milk production is predicted to be between 80% and 95%,[423] suggesting that during lactation basal metabolic rate should increase anywhere from 5% to 20% due to the energy cost of milk production.[424,425] Butte et al. elegantly showed in women that there is a direct correlation between milk production and total energy expenditure, consistent with the idea that metabolic rate is affected by the costs of milk production.[426] However, this effect on metabolic rate has not been observed in all studies, suggesting that the energetic efficiency of milk production may *vary* substantially between individuals or that peripheral tissues become more metabolically efficient to compensate for the increased energetic demand of milk production.

The efficiency of milk production is inherently linked to the type of macronutrient used to make milk lipid (Figure 46.17). Thus, it is energetically more expensive to produce milk lipid from carbohydrate or protein because as much as 25% of the energy must be expended to convert them to a free fatty acid precursor. On the other hand the energetic cost of producing milk lipid with preformed fatty acids derived from the diet or from endogenous stores is substantially less (<1–2%). For this reason the composition of the diet and the availability of preformed precursors can have a significant effect on the energetic requirements of milk production.

In addition, evidence suggests that the body can adapt in other ways to conserve energy for milk production (Figure 46.17). Three of the proposed adaptations are: (1) decreased physical activity[427–429]; (2) suppressed anabolic activity of adipose and muscle[430–432]; and (3) reduced nonshivering thermogenesis by brown adipose tissue (BAT).[427,433–437] With respect to BAT, falling leptin levels may coordinately explain the inhibition of BAT and the suppression of nonshivering thermogenesis during lactation.[404,427,438]

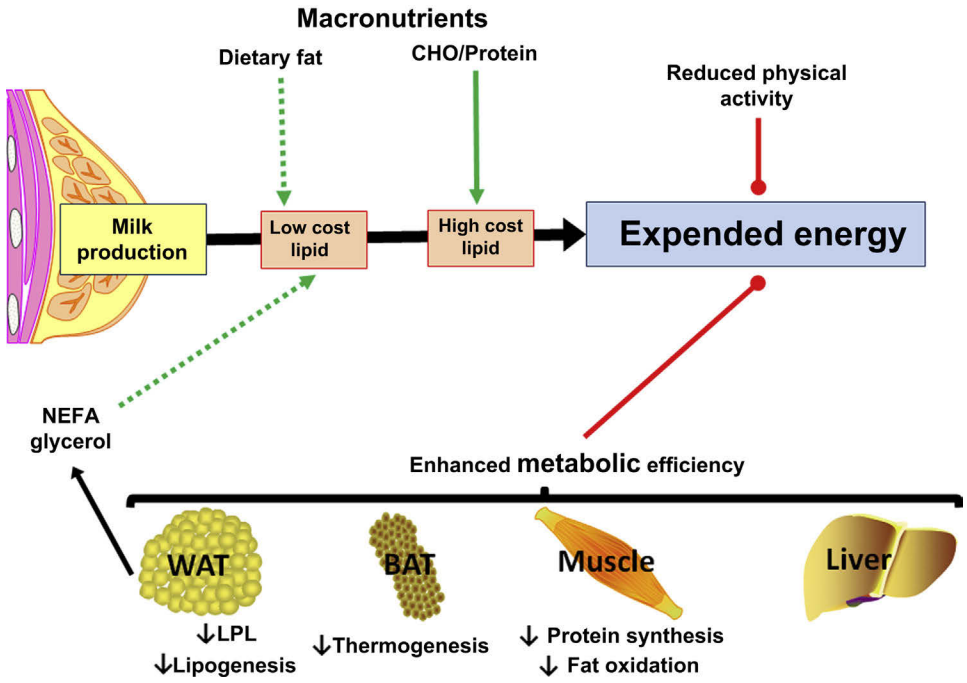

FIGURE 46.17 **The regulation of energy expenditure during lactation.** Milk production is energetically expensive. This cost includes not only the energy found in the milk constituents but also the energy that must be expended to produce and secrete the final product. Preformed fatty acids from the diet (exogenous) or mobilized from adipose tissues (endogenous) require relatively little expended energy to produce milk lipid. If milk lipid is made from CHO or amino acid precursors, these substrates must be converted to fatty acids via de novo lipogenesis, increasing the amount of energy needed to produce milk lipid. To conserve energy during lactation and maintain thermoneutrality, peripheral tissues become more metabolically efficient and physical activity declines. WAT = white adipose tissue; BAT = brown adipose tissue; NEFA = nonesterified fatty acids; LPL = lipoprotein lipase.

Dietary Fat versus De novo Derived Lipid: Effects on Milk Lipid Content

The positive energy imbalance resulting from the combined effects of hyperphagia and enhanced metabolic efficiency can provide the extra energy needed for milk production. The extra energy makes its way to the mammary gland in the form of carbohydrate, lipid, or protein, and may be trafficked through other tissues prior to being utilized by the mammary gland for milk production (Figure 46.18).

Breakdown products from hydrolysis of dietary lipids are taken up by the intestinal cells, re-esterified into triglycerides (TAG), and released in a lipoprotein called a chylomicron. Chylomicra are released into the lymphatic circulatory system and eventually enter the portal vein from which they are distributed through the blood stream to be utilized by lipoprotein lipase (LPL) containing tissues for oxidation or storage or synthesis by MECs into milk TAG. If there is very little fat in the diet, a larger portion of the lipid component of the milk must be produced from the available carbohydrate and/or protein, via a process called de novo lipogenesis. If this process occurs in the mammary gland, the fatty acids synthesized are limited to 8- to 16-chain saturated fatty acids, while in the liver and adipose tissues fatty acids of 16 carbons and longer are produced (Figure 46.18). Increased dietary fat has been shown to downregulate de novo lipogenesis in the mammary gland[439–445] and in turn reduce the proportion of medium chain fatty acids in the milk fat. As such, milk TAG composition reflects dietary fat composition.[446]

Endogenous Nutrients

In addition to the positive energy imbalance, endogenous nutrients are mobilized and directed to the mammary gland to meet the demands for lactation. PRL and β-adrenergic stimulation from the sympathetic nervous system provide critical signals for this shift in fuel trafficking. However, these signals have tissue-specific effects in the periphery. Fat stores in adipose tissue and liver (made via de novo lipogenesis or transported to these tissues from dietary sources[446]) are mobilized during lactation to provide milk fat substrates and energy for milk production.

Adipose Tissues: The Primary Source of Endogenous Energy

During pregnancy, ingested energy gradually accumulates in white adipose tissues (WAT) in preparation for lactation, stored as TAG in lipid droplets. During

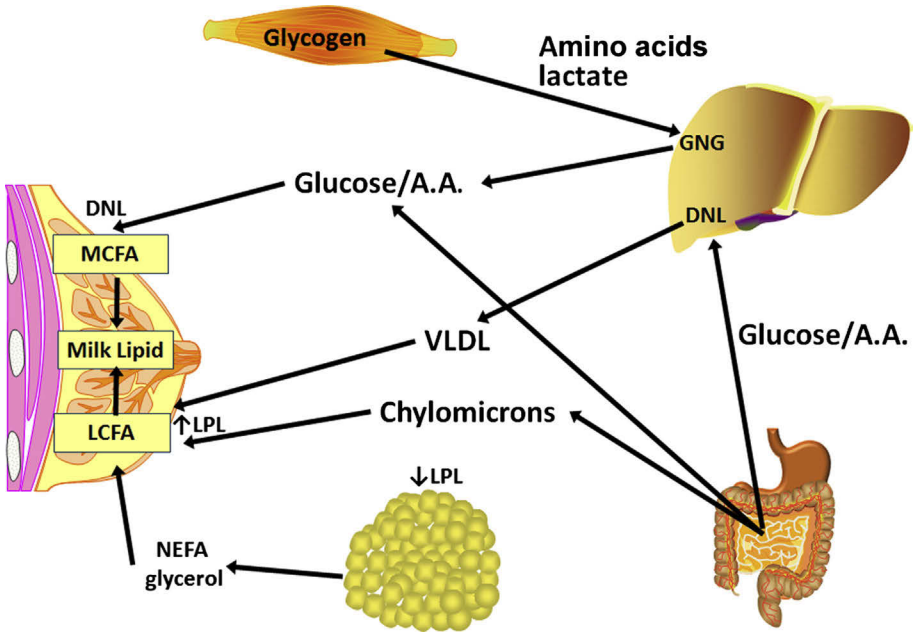

FIGURE 46.18 Exogenous and endogenous nutrients affect the composition of milk lipid. Dietary fats primarily enter circulation through the lymphatic system as triacylglycerol in chylomicrons, while the liver releases stored lipid in the form of VLDL. The differential expression of LPL in the mammary gland and adipose tissues leads to the trafficking of these neutral lipids toward milk production. Adipose tissue lipid is mobilized and trafficked to the mammary gland in the form of NEFAs. Glucose and amino acids, mobilized from endogenous stores or absorbed from the diet, are directed to the mammary gland as precursors for milk carbohydrate and protein. When in excess, the mammary gland and liver convert these precursors to fatty acids via de novo lipogenesis. The product in the mammary gland is primarily MCFA, while in the liver it is long-chain fatty acids (LCFA) and subsequently triacylglycerol. The types of fats that end up in milk lipid are a function of dietary fat (usually LCFA), the amount that is mobilized from endogenous stores (usually LCFA), and the location of DNL (MCFA, mammary gland; LCFA, liver). LPL = lipoprotein lipase; NEFA = nonesterified fatty acid; VLDL = very low density lipoprotein; DNL = de novo lipogenesis; A.A. = amino acid; MCFA = medium chain fatty acid; LCFA = long chain fatty acid.

lactation, this stored energy is then mobilized to provide fatty acid substrates for energy production and milk lipid synthesis.[77,431,446] Lipolytic enzymes, such as hormone-sensitive lipase and adipose triglyceride lipase, hydrolyze the lipid droplets to release the stored TAG. The released fatty acids and mono-acylglycerol are transported out of the cells into the blood. They bind to albumin and are circulated throughout the body.

This shift of WAT from an anabolic state during pregnancy to a catabolic state during lactation is in part due to the reversal of the low sympathetic tone that develops in this tissue during pregnancy. Shortly after parturition, the sympathetic tone in adipose tissues increases. The concentration of norepinephrine in WAT increases, stimulating β-adrenergic receptors on adipocytes, which become more responsive to the activation of lipolysis by cyclic AMP.[430,447–450] At the same time, insulin suppression of lipolysis in WAT and its ability to promote lipogenesis becomes impaired.[451,452] Under normal postprandial conditions or hyperphagia, elevated insulin will activate lipogenic pathways and inhibit lipolysis,[453] resulting in a net deposition of energy in WAT.[454,455] During lactation, impaired insulin sensitivity resulting from decreased expression[456,457] and responsiveness of IR[452,458,459] in WAT blunts the uptake and deposition of energy and diverts ingested energy to the mammary gland.[432,456,460] Evidence from both humans[461] and animals suggests that the PRL secreted after secretory activation may have direct effects on adipose tissues that suppress adiponectin secretion and impair insulin suppression of lipolysis.

In addition to mobilizing the stored lipid, the impairment in insulin signaling decreases the deposition of ingested carbohydrate and protein[430,449,462,463] so that these nutrients can be directed to the mammary gland. Glucose uptake and oxidation decline,[432,451,464,465] making the available glucose carbons for the production of milk.

Liver: An Alternate Source for Carbohydrate, Lipid, and Protein

During lactation, the liver serves as both a way station for endogenous nutrients and a metabolite interconverter to meet the nutrient needs of the mammary gland. Under postprandial conditions, excess nutrients accumulate in the liver and are stored until endogenous sources become less available. Unlike adipose tissues, hepatic lipid is released into the circulation in the form of very low density lipoproteins (VLDL). As such, this lipid becomes available to the mammary gland in much the same form as dietary fat (but via VLDL).

In addition to providing lipid, the liver converts metabolic intermediates into glucose in order to help to provide a regular supply of carbohydrate to the mammary gland. Skeletal muscle provides the precursors in the form of lactate[466] and amino acids[465] to support hepatic gluconeogenesis,[447,467] which increases during lactation.[424] Increased gluconeogenesis has been explained as a consequence of liver hypertrophy, a lower insulin-to-glucagon ratio in the portal vein, and intermittent hypoglycemia.[468] Hepatic glycogen also becomes a source for hepatic glucose production.[469] In humans, glycogenolysis may become the primary pathway for hepatic glucose production under fasting conditions.[470] However, there is evidence that the relative contributions of glycogenolysis and gluconeogenesis vary with species and metabolic state. Regardless, the end result serves to divert glucose to the mammary gland to meet the immense demands of milk production.

Improved Metabolic Control

Despite the fact that studies in animal models suggest that the insulin resistance of peripheral tissues helps to direct nutrients to the mammary gland, studies in humans indicate that this extension of pregnancy-associated insulin resistance resolves as lactation proceeds.[471] This improvement in metabolic control may lead to levels of insulin sensitivity that surpass that of nonlactating controls.[472] The mechanism of this improvement is not well understood, but it likely reflects the depletion of endogenous energy stores.

Trafficking Exogenous and Endogenous Nutrients to the Mammary Gland

Controlled in large part by the rate of milk secretion, active blood flow to the mammary gland increases the delivery of glucose, amino acids, fatty acids, and lipoproteins.[446,473,474] PRL also inhibits the expression of LPL in WAT,[475–477] while increasing LPL expression in mammary gland tissue.[477] This tissue-specific effect of PRL traffics triglyceride carried by chylomicra and VLDL away from WAT and to the mammary gland.

Glucose is the major substrate for synthesis of lactose and glycerol in the mammary gland during lactation. Glucose uptake in the mammary gland significantly increases during lactation, and although blood glucose may be somewhat elevated from lactation-induced hyperphagia, glucose uptake in the mammary gland is elevated independent of dietary supply.[465,478]

In rodents the proportion of long-chain fatty acids in milk is high during early lactation reflecting the mobilization of fat stores.[432] The increased availability of substrate for milk fat synthesis decreases the need for the mammary gland to produce its own lipid via de novo lipogenesis.[446] Endogenous fatty acids from WAT contribute 10–20% of the lipid used for milk fat production,[446] and mice mobilize up to 70% of their adipose reserves during lactation.[432] However, most of these endogenous stores are used up during the first several days of lactation.

Evidence for Impaired Lactation in Maternal Diet-Induced Obesity

Animal Models of High Fat Feeding and Maternal Diet-Induced Obesity

Rodent models of diet-induced obesity have historically been the most common models used to study the effects of obesity on pregnancy and lactation. Typically, obesity is induced by feeding a HF diet using either a cafeteria-style diet where rodent chow is supplemented with high calorie human snacks[439,443,479–481] or a semipurified diet using lard or vegetable oil as the primary fat source.[445,482,483] There are benefits and downfalls of both HF-feeding paradigms. Cafeteria-style diets are more representative of the human condition; however, the specific source of calories is not as well controlled as with semipurified HF diets. Additionally, when a rodent is left to choose its own food there is the potential for deficiencies in essential nutrients, which can have adverse effects on lactation independent of HF feeding.[484,485] Finally, it is important to consider the composition of the fatty acids used to induce obesity, as different types of fat substantially affect milk composition.[22,480,486]

Further, the conclusions that can be drawn from studies with diet-induced obesity models are confounded by the problem that HF diets themselves may have effects on lactation separate from effects of obesity. Typically, short-term exposures to HF diets are employed in lean animals to dissect these two effectors.[445,487–489] This approach is particularly useful in examining how HF feeding affects the various stages of mammary gland development. Separating the effects of chronic HF feeding from obesity is more challenging. To achieve this objective, some researchers have switched HF-fed, obese animals to a low fat diet during pregnancy, lactation, or both.[490] Such an approach, however, reflects an acute dietary change, rather than dissecting chronic HF feeding from the effects of obesity.

Diet-Induced Obesity Impairs Mammary Gland Development and Function

Obesity in dairy cows has long been known to impair milk production,[491] and rodent studies show similar effects.[443,492] When an HF diet was employed for 16 days prior to puberty in the mouse, one group found that the branching frequency and the width of mammary ducts were reduced along with the presence of abnormal myoepithelial cells in virgin mice.[492] In another study, lobuloalveolar development of the mammary gland was impaired when HF feeding was begun after puberty.[443] Morphologically, these authors reported abnormal side

branching and alveolar development by day 14 of pregnancy. In addition they showed a significant difference in mammary gland weight due to the increased size of the fat pad. Decreased pup weight, depressed milk synthesis genes, and the retention of large CLDs in the epithelium by day one of lactation suggested an inherent impairment in milk synthesis.[443] Together, these studies support the concept that diet-induced obesity can interfere with mammary gland function at different stages of development, but they fail to differentiate the effects of chronic HF feeding and obesity.

Unlike its effects on mammary gland development, the impact of HF feeding and obesity on milk production and composition has been relatively well characterized in rodents. The overall consensus is that diet-induced obesity impairs milk production early in lactation.[443,490,493] Observations of delayed pup growth and sometimes even death[479] within the first few days after parturition were an indication of decreased milk production and/or secretion. Additionally, milk composition is altered by HF feeding and obesity.[22,488] As mentioned previously, milk lipid composition is reflective of dietary lipids. However, it has also been shown that HF feeding can increase milk lipid content[480,490,493] as well as decrease milk protein.[443,480] However, in one study HF feeding during lactation actually led to decreased milk fat production.[445] Additionally, feeding conjugated linoleic acid or transfatty acids during lactation has resulted in suppressed milk fat production.[22,486,494] Therefore, the type of dietary fat may prove to be a determining factor in how milk composition is affected.

The effects of high-fat feeding and obesity during lactation on neonatal growth have also been inconsistent[441,443,444,495,496] with some studies reporting impaired neonatal growth linked to lactation defects[443,444] and others reporting increased neonatal growth correlating with an obese phenotype.[495,496] These inconsistencies suggest that the impact of HF feeding and obesity may vary with the stage of mammary development.

Evidence for Impaired Lactation in Humans with Diet-Induced Obesity

There is clear data indicating an increased risk for a failure of breastfeeding in obese women (BMI≥30).[497–500] Based on findings that obese white and Hispanic women are more prone to fail in initiating breastfeeding than normal weight women,[501,502] it has been proposed that obesity delays secretory activation[503,504] possibly by blunting PRL release in response to suckling.[505] Obesity is also associated with a shorter duration of breastfeeding.[497,506–509] Taken together, these studies indicate that obesity is an important risk factor for dysfunctional lactation. However, the biological basis for this increased risk has not been elucidated, and because of the current epidemic of obesity, it is an important area for further research.

MILK EJECTION

Secreted milk is stored in the alveoli and, to a limited extent, in the udder of ruminants. Milk ejection, necessary for milk delivery to the suckling, is effected by the contraction of myoepithelial cells whose processes form a basket-like network around the alveoli (Figure 46.19). Stimulation of nerve terminals in the nipple or teat produces impulses in spinal afferent nerves that reach the magnocellular neurons in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. The axons of these neurons run through the pituitary stalk terminating in the posterior pituitary[510] and releasing the nonapeptide, OT, when stimulated. Released OT is carried through the circulation to the lactating mammary gland where it interacts with specific receptors on myoepithelial cells, initiating a coordinated contraction that expels milk from the alveoli into the ducts and subareolar sinuses or udder. Milk ejection is essential for lactation, as shown by the observation that mice genetically deficient in OT have a severe lactation deficit.[511,512] Although milk ejection was the first recognized action of OT, the hormone and its receptors are found in many organs including the brain, where its release is regulated by a wide variety of peptides and hormones.[513] Methods are becoming increasingly available for assessing the effects of targeted mutations on this process. A particularly useful video illustrating techniques for

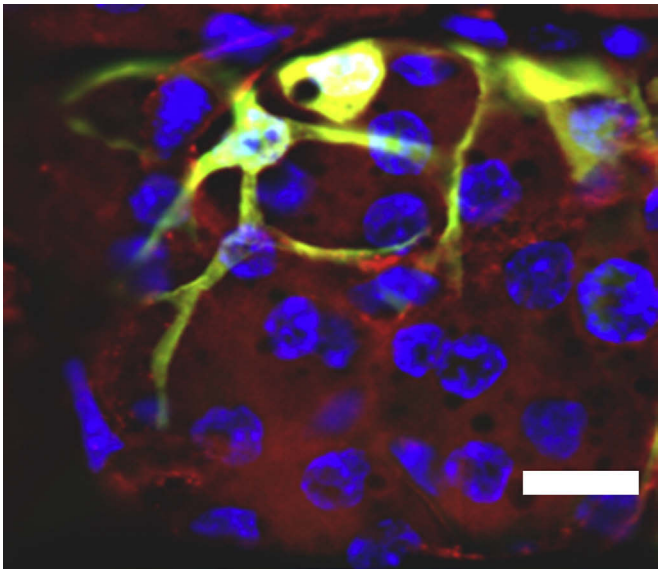

FIGURE 46.19 Myoepithelial cell in the mammary gland of a lactating mouse. This figure is reproduced in color in the color plate section. The cell has been transduced with adenoviral GFP (yellow) showing processes embracing the luminal epithelial cells (red). Nuclei stained with DAPI (blue). Scale bar 10 μm. *Source: From Russell T, Fischer A, Beeman N, Freed E, Neville MC, Schaack J. Transduction of the mouse mammary epithelium with adenoviral vectors in vivo.* J Virol *2003;77(10):5801–09.*http://dx.doi.org/10.1128/JVI.77.10.5801–5809.2003, *© 2003, American Society for Microbiology.*

assessing mammary development from whole mounts of tissue as well as an ex vivo technique for assessing myoepithelial contraction has recently been published.[514]

The Letdown Reflex

Figure 46.20 shows neurophysiological correlates of OT release in rats, humans, and cows. These processes are the subject of an excellent review by Armstrong.[516] The rat nurses her litter for about 30 min each hour. Letdown is delayed for at least 15 min after the attachment of the pups.[510,516] Thereafter, increases in mammary pressure corresponding to OT-induced milk ejection can be measured every 5–12 min (Figure 46.20(A) and (B)). In cows a more sustained release of OT has been measured (Figure 46.20(D)). In women, Cobo and colleagues[517] showed that ejection can be measured as a rise in pressure sensed with a small catheter placed in a mammary duct or noted subjectively by the mother as a "tingling sensation" in the breast prior to or shortly after the start of suckling. When milk was continuously pumped from the breast, these contractions lasted about 1 min and occurred with a frequency of 4–10 contractions per 10 minutes. When measured from blood samples drawn at 2 min intervals, the pulses of OT in the blood stream correspond to these contractile episodes. Unlike OT release in the rat, in women OT release begins prior to suckling in response to the cry of the infant or the mother preparing for the feed[518] (Figure 46.6(C)), indicating a psychological input to the magnocellular OT neurons.

The Neuroendocrinology of OT Synthesis and Release

OT holds the distinction of being the first naturally occurring peptide hormone to be synthesized,[519] a feat for which Vincent du Vigneaud received the Nobel Prize in Chemistry in 1955. OT release involves exocytosis of secretory granules stored in the posterior pituitary following a burst of impulses carried from the OT neurons in the SON or PVN.[510] Immunostaining techniques were used to show that OT is synthesized mainly in magnocellular neurons in these nuclei of the hypothalamus, separate from the vasopressin-synthesizing neurons.[520] Pulse chase experiments showed that OT is synthesized as part of a 30 kDa prohormone in the hypothalamus.[521] After cleavage to smaller subunits, the prohormone is packaged into secretory granules and transported down the axonal

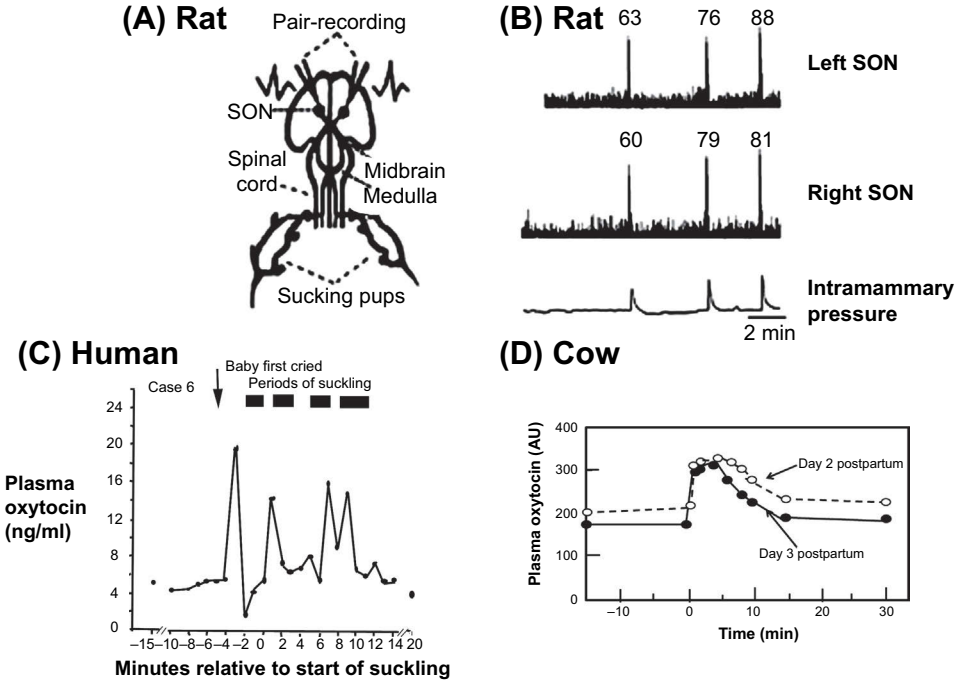

FIGURE 46.20 Oxytocin (OT) secretion. (A, B) Recordings from OT-releasing neurons in the anesthetized rat made simultaneously with recordings of intramammary pressure. Bursts of neuronal activity with firing rates indicated by the numbers above each peak are spaced at 5–12 min intervals. Neurons from both sides of the brain fire simultaneously leading to a pulse of OT release from nerve terminals in the posterior pituitary and a rise in the plasma OT level, followed shortly by a rise in intramammary pressure. (C) OT release in the woman during suckling. Plasma OT rises when the woman first hears the infant cry. Pulses of OT continue during suckling intervals. (D) OT release in the cow showing the prolonged pulse of OT in the plasma during milking on days 2 and 3 postpartum. *Source: (A, B) Used with permission from Wang YF, Negoro H, Higuchi T. Lesions of hypothalamic mammillary body desynchronise milk-ejection bursts of rat bilateral supraoptic OT neurones.* J Neuroendocrinol *2013;**25**(1):67–75. (C) Used by permission from Ref. 515. (D) Used by permission from Akers RM, Lefcourt AM. Milking- and suckling-induced secretion of OT and prolactin in parturient dairy cows.* Horm Behav *1982;**16**:87–93.*

processes of the SON and PVN neurons to the posterior pituitary. There it is further cleaved to OT and its binding protein, a 10 kDa neurophysin. The complex between OT and neurophysin is stable at pH 5.5, but dissociates at pH 7.4, freeing OT as the complex is released to plasma.[510,522]

The neurophysiology of OT release is complex and subject to regulation by steroid hormones and many other agents.[515,523] Critical to the appropriate release of OT during lactation is conditioning of the OT neurons during pregnancy.[523] OT release requires an increase in cytoplasmic Ca^{++}, both in magnocellular neurons and in nerve terminals; however, the Ca^{++} homeostatic mechanisms differ at the two locations with involvement of the endoplasmic reticulum in the dendrites, but not in the axonal terminals.[524] OT release is modulated by many agents including the sex steroids P4 and E2 as well as norepinephrine[515,523]; the mechanisms involved remain the object of intense study by neurophysiologists.[524,525] Of importance for lactation, endogenous opioid peptide systems inhibit release of OT at the magnocellular neurons in the hypothalamus and at their neurosecretory terminals in the posterior pituitary. Opioids likely also have an inhibitory effect on the cell bodies of afferent input cells that stimulate magnocellular neuron activity.[526]

Myoepithelial Cell Contraction

The processes of the myoepithelial cells lie within the basement membrane of the mammary alveolus and along the interlobular ducts. Autoradiographic studies showed that OT binding sites are similarly localized[527] and that there is 10-fold increase in the concentration of OT receptors in the rat mammary gland during pregnancy. The gradual increase in mammary OT receptor concentration contrasted sharply with the sudden increase in these receptors in the uterus on the day of parturition. The contractile response depends on interaction of smooth muscle alpha-actin (ACTA2) with myosin, and it has been found that lack of ACTA2 expression in null mice leads to severely impaired pup growth.[528] However, it appears that other pathways are involved in relaxation following this contractile response. Raymond et al.[529] found that conditional deletion of a laminin receptor, α3β1 integrin, from myoepithelial cells also led to low rates of milk ejection with impaired phosphorylation of focal adhesion kinase, an altered balance of elements of the Rac/Rho pathway and sustained phosphorylation of myosin light chain. These authors found that the lack of relaxation after OT-induced contraction in vitro could be rescued with constitutively active Rac or treatment with an inhibitor of myosin light chain kinase (MLCK). The authors suggest that α3β1 integrin stimulates Rac signaling that in turn inhibits MLCK activity, bringing about completion of the contraction relaxation cycle associated with milk letdown.

OT also appears to stimulate the growth of myoepithelial cells both in vitro and in vivo. When administered with E2 and P4 via implantable pellets into a virgin mouse mammary gland, it also enhanced myoepithelial differentiation of the cap cells surrounding the terminal end bud.[530] The potential role of OT in stimulating myoepithelial cell proliferation has recently been reviewed.[531]

The OT Receptor

The OT receptor is one of a family of nonapeptide hormone receptors that belong to the seven membrane spaning Rhodopsin-like Class I G-protein coupled receptors.[532] The human OT receptor has 389 amino acids and a core molecular mass of ~40–45 kDa that can be increased by glycosylation. The OT receptor gene has been cloned from a number of species[533,534] and a number of regulatory elements identified in the promoter including a palindromic estrogen response element and a half-steroid response element[534] that may be responsible for the E2-induced stimulation of OT receptor mRNA. In an elegant study, Olins and Bremel[535] found that OT stimulated the influx of extracellular Ca^{++} ions leading to phosphorylation of the myosin light chain in rat mammary myoepithelial cells. Influx of extracellular Ca^{++} ions regulated the duration of the response. There is also evidence that intracellular Ca^{++} stores are involved. Because the OT receptor is found in many locations, both in the brain and in organs such as the myometrium, heart, and peripheral nervous system, it is considered as a prime candidate for pharmacotherapeutic interventions.[536]

Cholesterol is an essential allosteric modulator of OT receptor function as is Mg^{2+},[533] although whether either plays a role in modulation of the activity of this receptor in the mammary gland is unknown. P4 has little effect on mRNA expression but exerts a powerful inhibitory action on receptor activity at a nongenomic level.[537] Whether this effect is mediated by interaction of P4 directly with the OT receptor as reported by Grazzini et al.[538] or by in some way altering the essential interaction of cholesterol with the receptor[539] remains to be elucidated. An interesting question is whether this interaction of cholesterol with the OT receptor plays a role in the elusive mechanism by which secretory activity is downregulated by P4 in late pregnancy.

OT and Maternal Behavior

OT has been implicated in a host of physiological and pathological functions such as stress management, maternal behavior, appetite, and social recognition. In the late 1970s, Pederson and Prange and others noted that intraventricular administration of

OT induced maternal behavior in virgin rats.[540] This observation led to extensive studies of the role of OT in maternal behavior (summarized in Chapter 51 and in Ref. 533). It has been found that OT neurons release the hormone not only from their axons but also from their dendrites in the hypothalamus where it acts on OT receptors that are widely distributed in the brain to decrease anxiety and appetite and facilitate social recognition.[541] It seems likely that these mechanisms are involved in the positive response to nursing observed by many mothers; in rats NMR images showed increased activity associated with OT in regions of the brain associated with mother–pup bonding.[542] The extensive literature relating OT and mood is beyond the scope of this chapter; a number of recent review articles are available for the interested reader.[536,541,543]

CONCLUSION

We are at a strategic junction in research on mammary development and function. Starting from a clear understanding of the systems physiology and biochemistry of milk secretion developed in the 1970s, in the succeeding decades the stages of mammary development as they coordinate with reproductive maturation have been well defined. We know the structures of the genes, mRNAs, and proteins that are involved in both development and the synthesis and secretion of milk components. More recently new technologies have allowed a finer structural definition of the minor components of milk, and we are on the cusp of understanding the role of oligosaccharides, cytokines, and glycosylated proteins in infant development. We have visualized the cell biology of the mammary cell at the level of both the light and electron microscope and now can marry structure and function using advanced immunocytochemical techniques to understand where proteins are located in the cell and how that localization varies with developmental stage and functional activity. Although the role of nutrition in milk secretion has occupied dairy scientists for decades, with the now clear effects of obesity on lactational competence in women, the whole body flux of nutrients and the dietary and hormonal regulation of these processes are coming to prominence. PRL and OT are no longer just hormones that promote milk synthesis and ejection, respectively; it is now clear that they have actions in the central nervous system that regulate nutrient intake as well as maternal-infant bonding. Our next steps are to delve more deeply into the protein–protein interactions that mediate both regulation of mammary development and milk secretion and to come to an improved understanding of how the nutrient and emotional demands of lactation fit into systemic metabolism.

A few of the many interesting questions that we must address in the next decades are:

1. Molecular regulation of mammary development
   - **a.** Ductal development: What regulates the temporal and special pattern of expression of the receptors for the hormones, particularly estrogens, and growth factors that regulate development? Is this pattern of expression established within the mammary stem cell compartment, and does it respond to environmental factors?
   - **b.** Alveolar proliferation and differentiation in pregnancy: While we know that changes in circulating P4, PRL, and PL drive these processes, what are the molecular pathways that lie between the receptors for these hormones and the genes whose expression is changed?
   - **c.** What are the roles of microRNAs, gap junction proteins, insulin, and other factors in secretory differentiation in pregnancy?
   - **d.** Could the inhibitory effects of P4 on milk secretion in late pregnancy be mediated by P4 interaction with the OT receptor in myoepithelial cells?
   - **e.** What are the membrane channels that mediate transfer of monovalent ions at both the apical and basolateral membrane? How do they change with development and what are the mechanisms by which they are regulated?
2. Involution
   - **a.** How do the many signaling molecules that have been shown to affect involution interact at the molecular level?
   - **b.** What is it that macrophages do to facilitate the involution process?
   - **c.** Do the tight junctions play a role in the increased Na^+ and Cl^- in milk during involution and mastitis, or does increased stress on the alveolar cells set in motion purinergic pathways that alter secretion and transport in alveolar cells? Could ATP and UTP be the elusive "feedback inhibitors of lactation" that have been shown to be present in both ruminant and human milk?[544,545]
   - **d.** In addition to macrophages, are there other immune cells and factors that interact with the involution process?
3. Nutrition, metabolism, and mammary development
   - **a.** Are SREBP and STAT5 the only transcription factors that drive alveolar development in pregnancy, and how is this process modulated by diet, obesity, or malnutrition?

   b. What is it that macrophages do to facilitate the involution process?
   c. What are the mechanisms by which nutrients are directed toward the lactating mammary gland for milk synthesis?
   d. How are the activities of organs such as the liver, digestive tract, and heart stimulated in lactating animals? Are hormones involved? If so, which ones, and by what mechanisms?
   e. What are the mechanisms that regulate appetite so that food intake is upregulated to meet the demands of lactation?
4. Systemic factors regulating milk secretion and ejection in lactation
   a. How do obesity and food intake interact with mammary gland development and milk secretion to alter lactation?
   b. Can we utilize the mRNA from the MFG to analyze the molecular basis of effects of obesity, emotional state, immune disorders, etc., on lactation in women?
   c. What are the mechanisms by which infant gender and maternal emotional states alter milk secretion and composition?
5. Interactions between the immune system and mammary development and function
   a. What are the details of the interaction of dIgA with its receptor and the cleavage event that releases secretory IgA into the milk?
   b. More recent evidence indicates that immune cells such as macrophages and eosinophils are necessary for both ductal development[546] and involution.[183] What other immune interactions promote or impede mammary development, particularly during pregnancy?
   c. Are there specific immune reactions that link maternal diseases, obesity, and metabolic disorders to lactation?
6. The maternal and infant microbiome
   a. What are the critical nonnutrient components that support normal neonatal development, and how do they interact with the infant gut and immune system?
   b. What components of milk foster development of the infant microbiome, and how is their secretion into breast milk regulated?
   c. If microbes from the mother are actually transferred to the infant through the milk, how do they get across the mammary epithelium? Is the process selective?
7. Lactation and breast cancer
   a. How does breast-feeding before the age of 30 suppress breast cancer risk?
   b. What are the mechanisms by which pregnancy promotes breast cancer risk?

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CHAPTER

47

Sexual Differentiation of Brain and Behavior

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INTRODUCTION

The vast majority of vertebrates come in two sexes: male and female. Because at most half of the individuals (i.e., females) in a sexually reproducing species ever give birth, this severely reduces their reproductive potential, something that has been referred to as "the cost of producing males".[1] The textbook explanation for why sexual reproduction is so ubiquitous, despite this large cost, is that sexual reproduction allows for genetic variability, which can be an advantage in a highly variable world. There is, however, surprisingly little direct evidence to support this theory, and there is some evidence to the contrary.[2] Another, not mutually exclusive theory proposes that sexual reproduction provides a mechanism for minimizing the number of mutations within a population, most of which will be neutral or mildly deleterious.[3] Yet another theory states that the rapid evolution afforded by sexual reproduction allows a population to outrun pathogens, or outcompete other organisms, thereby holding on to a niche that it already occupies.[4] This "Red Queen Hypothesis" is named after a character in Lewis Carroll's *Through the Looking Glass* who has to run as fast as she can just to stay put. Remarkably, none of these three leading theories have as yet been accepted as fully satisfactory explanations for the existence and pervasiveness of sexual reproduction among eukaryotes.[5] As Margulis and her colleagues gently mocked some years ago, "Sexual reproduction is still a waste of time and energy".[6]

In contrast to our fuzzy understanding of why, exactly, sex evolved, its biological consequences are often clear. To put it briefly: *where there is sex, there are sex differences*. In practically all cases where reproduction involves males and females, organisms can be easily distinguished based on male- and female-typical anatomical, physiological, and behavioral characteristics, which we will refer to as masculine and feminine traits in this review. For example, to reproduce, males typically generate an abundance of small germ cells (sperm), whereas females make many fewer, large cells (ova). Males and females also typically display different behavioral strategies, the ultimate explanation of which is to maximize the chances that their germ cells will fuse with those of the opposite sex to produce viable offspring. In addition to differences in morphology, males typically show one set of reproductive behaviors, whereas females show another. Such differences have presumably been selected for whenever they enhanced the chances of successful mating. Depending on the species, males and females may also show differences in behaviors and cognitive functions that are not directly related to reproduction; such traits may indirectly contribute to the reproductive potential of the organism or may simply be pleiotropic consequences (essentially, side effects) of the sexual differentiation of reproductive processes.

Sex differences in behavior have inspired researchers to look for underlying causes in the brain. Not all of this research has withstood the test of time. In the nineteenth century, for example, men's brains were reported to be heavier and more symmetric than those of women, differences that were presumed to reflect differences in intellect.[7,8] Even though recently sex differences in gross structure of the human brain that cannot be explained just by differences in body weight have been confirmed,[9–12] this line of research did not get much traction, probably at least in part because of the dubious interpretations. Two lines of animal research rekindled interest in sex

Knobil and Neill's Physiology of Reproduction, Fourth Edition
http://dx.doi.org/10.1016/B978-0-12-397175-3.00047-8

differences in the brain in the second half of the twentieth century, one physiological and the other behavioral. The first started with a classical experiment by Harris and Jacobsohn done in the early 1950s, aimed at clarifying why females generate surges in gonadotrophic hormones (associated with ovulation), whereas males exhibit more tonic release.[13] They showed that female rats with male pituitary transplants placed under their hypothalamus still exhibited ovarian cycles.[13] This important demonstration disproved the idea that pituitaries or gonads were driving the sex difference in gonadotropic hormone release and pointed instead to sex differences in the brain, specifically the hypothalamus. A couple of years later, Barraclough et al. showed that neonatal injections of testosterone led to infertility in female rats.[14,15] After demonstrating that the anterior preoptic area of the hypothalamus drives cyclicity in gonadotropic hormone release, they suggested that "the anterior preoptic area is undifferentiated at birth with regard to its subsequent control of gonadotropin secretion", and that, in the absence of androgen, the anterior preoptic area differentiates to sustain cyclicity (as in females), whereas in the presence of androgen it "becomes refractory to both intrinsic and extrinsic activation, and the more tonic type of male gonadotropin secretion is observed."[16] Although this paper related to the function rather than structure of the brain, it suggested a fertile region to look for sex differences in brain structure.

Many previous reviews have catalogued sex differences in the mammalian brain,[17–26] and we will not attempt to repeat that here. Instead, we will focus on mechanisms for generating neural sex differences, and will highlight new areas of investigation in this field. We also will consider, in some depth, the function of neural sex differences. Although some sex differences in the nervous system can be related to differences in physiology and behavior, we find that in most cases the extent to which neural structure relates to function remains unclear.

A BRIEF HISTORY AND DEFINITION OF TERMS

Sex differences in physiology and behavior were recognized long before scientific attempts were made to study the causes and consequences of such differences. One of the first studies, often cited as a foundation of modern research in the differentiation of the brain and behavior, built on something farmers had known for centuries: that castrating a male chick early in life produces a capon, an animal lacking many of the masculine traits of the normal, gonadally intact rooster. In 1849, Berthold showed that if a castrated male chick was given a testis transplant, it developed the typical male, rooster-like phenotype.[27] Not only did those animals look like intact roosters, but they crowed and postured like roosters as well, instead of showing the "feighaft" (timid) behavior common to capons. This result led to the important insight that substances produced by the testes caused the behavioral differences between roosters and capons. Where these substances (which we now know are gonadal hormones) acted to change behavior was not really addressed, however, for another 100 years.

The idea that sex differences in gonadal hormone levels early in life cause lasting sex differences in behavior was first clearly stated by Phoenix et al., who found that injecting pregnant guinea pigs with testosterone permanently masculinized the sexual behavior of their female offspring.[28] Even though these authors only speculated that neural tissue (i.e., the brain) itself was sexually differentiated, their hypothesis—that during development, steroid hormones have permanent or, as they labeled it, "organizational" effects, as opposed to the transient, or "activational", effects that occur later in life—remains a fundamental principle of sexual differentiation of brain and behavior (as discussed in this chapter). Over the last 50-plus years, the "organizational hypothesis", as this idea was soon dubbed, has guided numerous researchers in their study of behavioral and neural sex differences, and their causes and consequences (Figures 47.1 and 47.2). The fact that the organizational hypothesis was developed to account for sex differences in behavior may be why, even today, a search in PubMed for papers with "sex differences" in the title turns up an inordinate number of studies in those disciplines related to behavior: neuroscience, psychology, and psychiatry.

Use of the terms "organizational" and "activational" to distinguish between permanent and transient effects of gonadal steroids, respectively, has had enormous heuristic value, and the organizational hypothesis was justifiably honored with a special issue in *Hormones and Behavior*,[29] and an editorial in *Endocrinology*[30] on the occasion of its 50th birthday in 2009. Nonetheless, we would like to argue that it may be time for a change. The term "programming" is currently used in most other fields of biology to describe lasting effects of endogenous as well as exogenous factors on biological functions, including factors involved in sexual differentiation of gonadal tissue (see, e.g., Ref. 31). As there are no reasons to assume that differentiating effects of gonadal hormones on brain function differ in principle from other programming effects, holding on to the term "organizational" may become a hindrance to communicating with scientists in other fields and in uncovering general principles that underlie long-lasting effects of gonadal hormones on the development of the brain and behavior. Likewise, as the term "activational" is usually used only in the context of the "organizational hypothesis", we will emphasize the more widely understood term "acute effects" throughout this review.

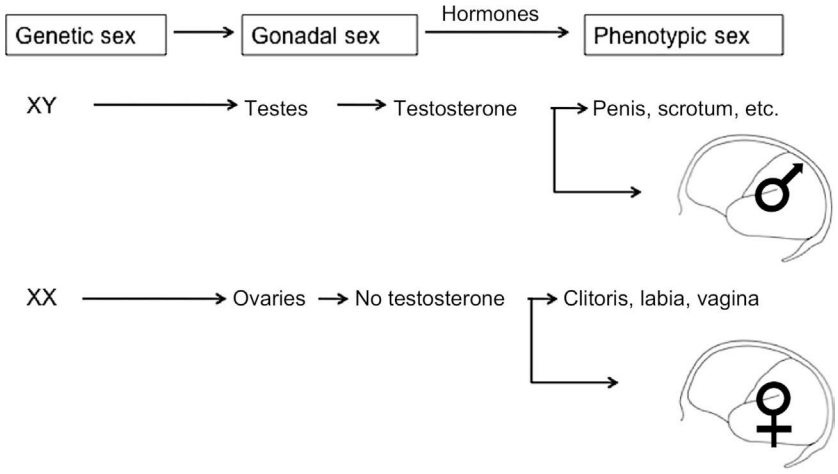

FIGURE 47.1 The organizational hypothesis, originally proposed by Phoenix and collegues and subsequently amended by many others, emphasizes the role of testosterone in sexual differentiation of the body and brain. In genetically male mammals (XY), the undifferentiated gonads develop into testes, which then secrete testosterone. This testosterone acts as an androgen or after conversion to an estrogen to masculinize the body and brain. In genetic females (XX), the undifferentiated gonads develop as ovaries; testosterone is not produced, and the female phenotype results. Such "organizational" or "programming" effects of testosterone can account for many of the known sex differences in the neurobiology and behavior of mammals. *Source: Original artwork by S. Marc Breedlove.*

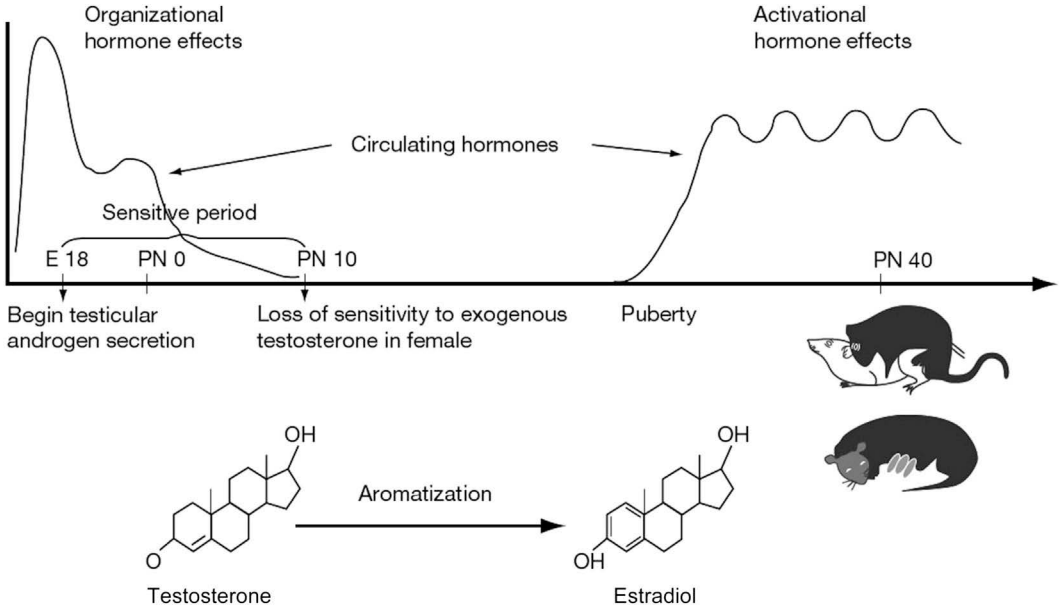

FIGURE 47.2 The organizational–activational hypothesis of steroid hormone action as it applies to laboratory rats, a species intensively studied in the sexual differentiation field. A perinatal sensitive period is operationally defined as the onset of gonadal steroid synthesis in males, beginning prenatally around embryonic day (E) 18, followed by a second surge on the day of birth (postnatal day (PN) 0) and the loss of sensitivity of females to exogenous steroid administration, which is usually around PN10. During the sensitive period, steroids are said to have organizational or programming effects because they are largely permanent. The organized, or programmed, neural substrate is then acted upon by adult, circulating steroid hormones and may cause the expression of sexually dimorphic behaviors, such as sexual behavior, or may coordinate behavior so that it is similar in both sexes, such as parenting behavior. Alternatively, the acute, or activational, effects of steroids in adults may be entirely independent of organizational–programming effects and may induce temporary sex differences. Testosterone can be converted to one of several estrogenic hormones via a process known as aromatization. Many of the effects of testosterone on the brain and behavior of rodents are secondary to its aromatization to estradiol, especially in rodents. *Source: Adapted with the permission of Elsevier from Ref. 24.*

This chapter will focus especially on the process by which male and female brains become different, first by identifying the roles of gonadal hormones, sex chromosomes, and environmental factors in brain differentiation. Next, we discuss the molecular and cellular targets of these factors, as well as the role of context in the expression of sex differences. Although much of the evidence in the foregoing sections focuses on animal models, we follow with a section on sexual differentiation of the brain and behavior as it applies to humans and conclude by presenting some emerging concepts and future directions in the field.

AGENTS OF SEXUAL DIFFERENTIATION

Perhaps the most important strategy that has been brought to bear on the question of how sex differences in behavior arise has been drawing upon lessons learned about how sex differences in body structure arise.

This strategy was made explicit in the aforementioned groundbreaking paper by Phoenix et al.[28] and has been fruitfully followed since. Taking cues from sexual differentiation of the periphery not only offers testable hypotheses but also helps constrain the number of possibilities that one might consider, or at least suggests which possibilities are more likely to pay off. As we'll see, as of this date, the approach has been very successful. Many sex differences in behavior, in nonhuman animal models at least, have been successfully manipulated by altering one or another factor that has been shown to underlie differentiation of the body. This success itself suggests that natural selection has hit upon a limited number of mechanisms to accomplish sexual differentiation, of either the body or the brain. Thus, when one chances upon any sex difference in behavior, one can systematically test each of the factors shown to underlie sexual differentiation of the periphery to see whether that same factor underlies sexual differentiation of the behavior in question.[32]

We can categorize the factors that have been found to affect the development of sex differences in the body into three classes.

Sex Chromosomes

Some sex differences result from differences in the inheritance of genes, specifically chromosomes that are found exclusively in one sex. Among vertebrates, this mechanism is especially important in mammals, in which only males inherit a Y chromosome and are therefore heterogametic, and birds, in which females are the heterogametic sex.[33] In these species, sex chromosomes trigger a molecular cascade that ultimately determines whether the embryonic gonad, which initially is undifferentiated or "bipotential", will develop as an ovary or a testis.[34] In mammals, the *sex-determining region of the Y chromosome* (*Sry*) gene, in cooperation with several other downstream genes, induces the indifferent gonad to develop as a testis.[35,36] In the absence of *Sry*, as in most XX individuals, the indifferent gonad will develop as an ovary.

We will see that some sex differences in behavior also appear to develop under the direct influence of genes on the sex chromosomes, including possibly *Sry* itself (see the section The Role of Sex Chromosomes). Note that in other vertebrates, including many reptile and fish species, there are no sex-specific chromosomes or genes. Instead, environmental factors, such as the temperature at which the egg is incubated, regulates the expression of genes that trigger development of either an ovary or a testis.[37,38] The gonads then arrange for individuals of the two sexes to be exposed to different levels of steroid hormones, which accounts for many secondary sex characteristics.

Gonadal Hormones

Regardless of how the fate of the bipotential gonads is determined, much of the sexual differentiation of the body is directed by hormonal secretions from the gonads. Paraphrasing Jost's classic formulation, genetic sex determines gonadal sex and gonadal sex in turn determines phenotypic sex (see Figure 47.1).[39] Genetic sex affects gonadal development via differential gene expression, but gonadal sex affects phenotypic development via hormone release. Of course, any hormone that affects the development of an organ, including the brain, is likely to do so by directly or indirectly regulating gene expression in that target organ, so we may think of the gonads as using hormones as intermediaries in regulating gene expression in other tissues.

In mammals, several testicular secretions are crucial for development of a male phenotype. Anti-Müllerian hormone (AMH; also called Müllerian-inhibiting hormone), is secreted by Sertoli cells and, as the name implies, suppresses development of the precursors of the female duct system, the Müllerian ducts, thereby averting development of the oviducts, uterus, and inner vagina.[40] It is the earliest product known to be secreted by the fetal testis; in mice, this occurs from embryonic day E12.5 until about 1 week postnatally.[41,42] In females, AMH is expressed by granulosa cells in the ovary, but only from postnatal day 6 onward.[41] Other, often overlooked testicular products play a role as well. For example, insulin-like factor 3 (INSL3) triggers the shortening of the gubernaculum, a ligament that at one end connects to tissue that will become the scrotal wall and at the other end connects to the testis. Shortening of the gubernaculum pulls the testis through the inguinal canal into what will become the scrotal cavity. Thus, mice with a mutation in either INSL3 or its receptor develop cryptorchidism.[43,44] Although AMH and INSL3 have traditionally been thought to play roles only in sexual development of the periphery, several recent studies suggest a role for AMH in sexual differentiation of the brain and behavior in both mice and humans (Figure 47.3).[45–47]

The main testicular products directing masculine development form a class of steroid hormones known as androgens, including the principal testicular secretion, testosterone (Figure 47.2). Androgens are responsible for "virilizing" Wolffian duct structures, precursors of the male duct system, and for shaping the external genitalia into a masculine form. They are also responsible for almost all male secondary sexual characteristics.

Nearly all work on sexual differentiation of the brain and behavior has focused upon the notion that testosterone or its metabolites are responsible for masculinizing males and, to a truly remarkable extent, this has turned out to be the case. In the accounts that follow, it will almost always be the action of testosterone, acting

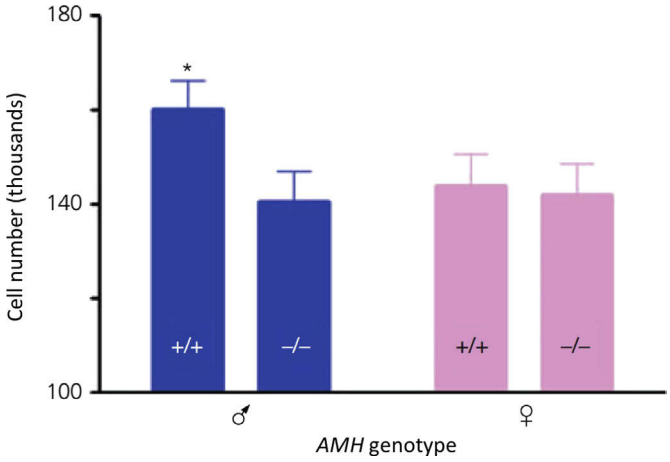

FIGURE 47.3 Anti-Müllerian hormone (AMH) influences some sex differences in the brain. This figure illustrates the number of Purkinje cells in the cerebellum in wild-type and AMH-knockout male and female mice. There is a male bias in the number of Purkinje cells in wild-type mice that is eliminated in the AMH knockouts, indicating that this particular sex difference depends on AMH. These findings suggest that testosterone is not the only gonadal hormone that is important for sexual differentiation of the brain. Significantly different compared to female and *AMH−/−* male mice. *Source: Adapted with the permission of John Wiley and Sons from Ref. 45.*

upon androgen receptors (ARs), (following conversion to estrogen) estrogen receptors (ERs), or both ARs and ERs, that shapes the developing brain into a masculine configuration and masculinizes behavior. Indeed, this outcome is so pervasive that exceptions to the rule will command some additional consideration in this chapter (see the section The Role of Sex Chromosomes).

Environmental Factors

The category of environmental factors is not only broad but also, for all practical purposes, infinite. But in terms of the sort of factors that might play a role in sexual differentiation of the nervous system, the list of those that have actually been examined by scientists is not quite so long. In fact, those other factors can be roughly divided into (1) environmental factors that mimic or interfere with hormonal signaling systems (e.g., endocrine disruptors), and (2) social influences.

There are some studies of nonhuman species demonstrating that social influences affect sexual differentiation of behavior, and even a few that demonstrate social influences on nervous system structure or function.[48–50] What makes these few studies especially noteworthy is the possibility that they may relate to social influences on the sexual differentiation of brain and behavior in our own species. As the gender roles of humans differ considerably across cultures and have changed appreciably across history, it is obvious that society exerts powerful influences on sex differences in human behavior.

This social influence on gendered behavior in humans appears to begin at birth. In a series of studies that are sometimes referred to as "Baby X" experiments, adults are handed a newborn infant, whose sex is clearly communicated, and then observed to see how they interact with the child. On average, adults of either sex spend more time looking into the baby's face and talking to the baby if they believe that the child is a girl than a boy. They are also more likely to bring a doll into play with the child if they believe it is a girl.[51] Conversely, they interact more physically, moving the child around through the air, if they believe the child is a boy than a girl.[52] These differences in adult behavior are not cued by the infant's preferences, because the genuine sex of the baby has no discernable effect on the adults' behaviors.[52] It is impossible to rule out the possibility that differences in how adults interact with babies, based on what they perceive as the child's proper gender, play a role in the later unfolding of sex differences in verbal ability (favoring females) and rough-and-tumble play (favoring males), among others.

Nevertheless, we will see evidence that hormones play a role in sexual differentiation of some human behaviors. Evaluating that evidence requires us to consider whether social influences may have in fact played a role. For example, as boys engage in more rough-and-tumble play than do girls, then the question is whether that sex difference comes about via the same mechanisms that direct sexual differentiation of the body, or via social interactions with other humans who encourage physical activity more often in boys than in girls, and encourage verbal social interactions more often in girls than in boys. Perhaps not surprisingly, both biological and social factors play a role and in some cases may reinforce each other.[53,54]

THE ROLE OF SEX CHROMOSOMES

The role of gonadal hormones in sexual differentiation was known long before the genetic basis of sex was firmly established. As mentioned in this chapter, the work of Berthold in the nineteenth century, and Jost and others in the first half of the twentieth century, highlighted the crucial role of the testes in masculinization. It was not until 1959 (interestingly, the same year as the groundbreaking work by Phoenix, Goy, Gerall, and Young, also mentioned here) that the Y chromosome was established to be testis determining in mammals,[55,56] and it would take an additional 30+ years before the testis-determining gene, *Sry*, was identified.[35] This may help to explain why, until quite recently, the prevailing view has been that although the sex chromosomes determine which gonad develops, it is hormones from the gonad that do the rest of the work as far as sexual differentiation

is concerned. It is now clear that this idea is in need of revision as evidence for direct, gonad-independent effects of sex chromosomes has accumulated. On the basis of this new evidence, it may be time for a revised, unified view of sexual differentiation that recognizes the primacy of genetic factors and is, as one author has put it, less "gonad-centric" (Figure 47.4).[58]

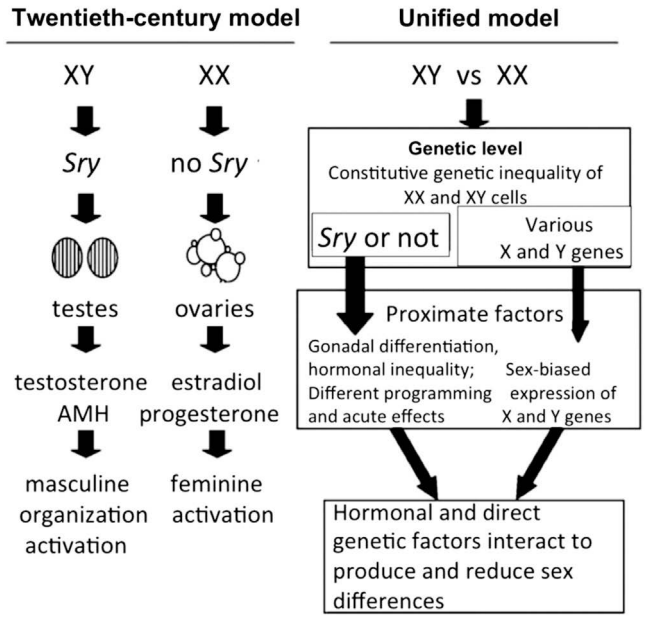

FIGURE 47.4 Contrast between the predominant twentieth-century model to explain sex differences in the phenotype of tissues, with a revised, unified model. In the twentieth-century model, the sexual differentiation of the gonads is ascribed to the male-specific effect of the Y-linked gene *Sry*. Once the gonads have differentiated, they secrete different sex steroid hormones. As described in Figures 47.1 and 47.2, these act on diverse tissues (e.g., the genital tracts and brain) in the fetal and neonatal male to cause masculine patterns of development resulting in permanently sexually differentiated substrates. Later in life, ovarian and testicular hormones act differentially on those substrates to create further sex differences in phenotype. In contrast, the unified model recognizes that *Sry* and other (to be identified) X and Y genes occupy the same primary logical level because they are all unequally encoded by the sex chromosomes in males and females. Some X and Y genes act in a sex-specific manner, on the gonads and other tissues, to cause sex differences in XX and XY cells. *Sry* plays a dominant role by setting up the lifelong sex difference in secretion of gonadal hormones, which have organizational–programming and activational–acute effects on the brain and other tissues. Because of the independent sex differences in sex chromosome genes and in hormonal secretions, the various sex-specific factors interact in one of several ways. Their effects are synergistic (as, e.g., when Y factors and testicular testosterone both push the male's tissues to function differently than in females), or they counteract each other to reduce sex differences (e.g., when the female-specific process of X inactivation shuts down one X chromosome in each female cell to counteract the female bias in X gene expression that would otherwise occur). With minor modification, the schema shown here can apply equally to birds or other groups that have a constitutive sexual imbalance of sex chromosome genes, by substituting species-appropriate sex determining gene(s) for *Sry*. *Source: Reproduced with permission of Elsevier from Ref. 57.*

Sex chromosome complement is the only thing that differs between XX and XY zygotes. As described here, one very important consequence of this genetic difference is the specification of testis development by the Y chromosome gene, *Sry*. And, indeed, a host of sex differences follow as a result of testis formation and exposure to the hormones produced by the testes. However, some sex differences are independent of the gonads. It has long been known, for example, that in tammar wallabies, differentiation of the scrotum versus pouch is controlled by the number of X chromosomes and not by gonadal hormones.[59] There is also more recent evidence that gene expression in very early XX and XY embryos differs prior to the time of gonad differentiation. For example, nearly one-third of the actively expressed genes in the bovine embryo may be expressed differently in males and females as early as the blastocyst stage,[60] that is, well before the formation of ovaries or testes.

Another approach to examining gonad-independent effects on sexual differentiation has been the use of animals in which no testes or ovaries form. In mice lacking the gene for steroidogenic factor 1 (SF1), for example, the undifferentiated gonads initially form, but then regress at an early stage.[61] Female duct structures and external genitalia are present in XY SF1-knockout animals, indicating that, indeed, there has been little or no exposure to testosterone or AMH.[61] These mice therefore provide an opportunity to examine the development of XX and XY animals in the absence of hormones produced by their own gonads. A caveat to this model is that the adrenals also do not form (animals are kept alive by glucocorticoid replacement at birth and later adrenal transplants), and there is a disruption in the development of at least one brain region, the ventromedial nucleus of the hypothalamus,[62] which is independent of the effect of the SF1 gene knockout on peripheral tissues.[63] Abnormalities in the brain and behavior of SF1 knockouts therefore could be due to a number of causes. Nonetheless, since SF1 gene deletion prevents the formation of the gonads, adrenals, and VMH in both XX and XY mice, any sex differences remaining in SF1-knockout mice likely reflect sex chromosome complement.

As predicted by the traditional view of gonadal steroid-dependent sexual differentiation, many sex differences in the body, brain, and behavior are eliminated in SF1-knockout mice. For example, size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) is normally larger in males than in females of several species (Table 47.1), due to the actions of testicular steroids acting early in development. The SDN-POA is completely female-like in SF1 knockouts, regardless of whether they are XX or XY,[153] confirming an essential role for the gonads in the differentiation of this brain region. There are exceptions, however. Male wild-type mice have more of the enzyme neuronal nitric oxide

TABLE 47.1 Examples of Several Well-Studied Sex Differences in the Rat Brain and Their Underlying Causes

Neural Area	Nature of the Sex Difference in Rats	Likely Cause(s) of the Sex Difference	Other Species Examined
Preoptic area of the hypothalamus (POA)	Males have more dendritic spines on neurons, more complex morphology of astrocytes; and more activated microglia than do females.[64–66]	Testosterone acts perinatally after conversion to an estrogen to program all three differences. Estradiol induces prostaglandin-E_2 synthesis to alter dendritic spines and microglia.[64–66]	n/a
Sexually dimorphic nucleus of the preoptic area (SDN-POA)	Males have larger volume and larger neurons and more neurons than females in this densely staining subregion of the POA.[67]	Testosterone acts perinatally after conversion to an estrogen to prevent cell death in males.[68–74]	Homologous cell group also larger in male mice, gerbils, guinea pigs, ferrets, hyenas, sheep, monkeys, and humans.[75–85]
Spinal nucleus of the bulbocavernosus (SNB)	SNB motoneurons are larger and more numerous in males than females. These motoneurons reside in the spinal cord and innervate striated muscles of the phallus.[86]	Testosterone acts via androgen receptors to prevent the death of SNB cells perinatally. The hormone likely acts at the target muscles, with SNB cells rescued indirectly.[87–94]	Similar sex difference in most other mammals studied to date, including mice, gerbils, dogs, hyenas, monkeys, and humans.[95–99]
Anteroventral periventricular nucleus (AVPV)	Females have larger volume and more neurons than males in this hypothalamic nucleus located near the rostral extreme of the third ventricle.[100]	Testosterone acts perinatally after conversion to an estrogen to increase cell death in males.[73,101–104]	Similar sex difference in mice.[105,106]
Bed nucleus of the stria terminalis, principal nucleus (BNSTp)	Males have larger volume and more neurons than females in this relatively large forebrain structure.[107,108]	Testosterone acts perinatally after conversion to an estrogen and via estrogen receptor α to prevent cell death in males. Androgen receptors also play a role in masculinization of BNSTp volume.[74,78,107,109–112]	Homologous cell group also larger in males in guinea pigs, mice, and humans.[78,105,113]
Medial amygdala, posterodorsal division (MePD)	Males have larger volume, and more neurons and astrocytes, as well as larger neurons and astrocytes, than females. These sex differences are hemisphere dependent.[108,114,115]	Dependent on both androgens and estrogens acting in adulthood; testosterone increases MePD volume in adult males.[115–117]	Similar sex difference in mice.
Vasopressin expression	Males have more vasopressin-expressing neurons in the bed nucleus of the stria terminalis and medial amygdala, and denser projections to target regions, than do females.[118–121]	Testosterone acts perinatally via both androgen and estrogen receptors to increase the number of vasopressin neurons. Also a direct effect of the Y chromosome on vasopressin cell number. Gonadal hormones influence the decision to make vasopressin, rather than by altering cell death, and are required in adulthood for maintenance of vasopressin expression.[121–132]	Similar sex difference in most other vertebrates studied to date, including frogs, lizards, birds, and mammals; too numerous to list here but reviewed elsewhere.[133,134]
Kisspeptin expression	Females have many more cells expressing the peptide kisspeptin in the AVPV than do males.[135]	Testosterone acts perinatally after conversion to an estrogen; cell death does not seem to be involved in establishing the sex difference. Gonadal steroids also regulate expression in adulthood.[135–137]	Similar sex difference in humans and animals as diverse as mice and fish, but no sex difference in lizards.[138–142]
Estrogen receptor	Females have more estrogen binding (or, in later studies, more estrogen receptor alpha protein or mRNA) in the POA than do males.[143–146]	Dependent on programming and acute effects of gonadal steroids; testicular steroids downregulate receptor expression in males.[144,147,148]	Similar sex difference seen in mice and voles.[149–152]

synthase in the anteroventral periventricular nucleus (AVPV) than females, and this sex difference persists in SF1-knockout mice.[153] The existence of a sex difference in the knockouts (i.e., in the absence of gonads) suggests a contribution of sex chromosomes to the development of this neural trait.

For some traits, a sex difference seen in wild-type mice is reduced but not eliminated in SF1 knockouts, suggesting that both gonadal steroids and sex chromosomes may contribute to the dimorphism. For example, when gonadectomized in adulthood and treated with estradiol followed by progesterone, wild-type female mice show much more female sexual behavior than do similarly treated wild-type males.[154] Although this female sex behavior is reduced in both XX and XY SF1-knockout mice, a sex difference nonetheless persists: XX SF1 knockouts exhibit higher lordosis quotients and receive more intromissions during testing than do XY knockouts (Figure 47.5).[155] Again, a contribution of sex chromosome genes, independent of their effects on the gonads, is suggested but not directly proven by this finding. Additional results using this model have recently been reviewed.[156]

A second and very effective mouse model used to directly test for the effects of sex chromosomes on sexual differentiation is the so-called Four Core Genotypes (FCG) model.[157] In this model, the *Sry* gene is deleted from the Y chromosome (designated Y-, or Y-minus) and inserted on an autosome. Whether an animal is XX or XY, if it inherits the autosomal *Sry* gene, testes will form and testicular hormones will be produced. By crossing wild-type XX females with XY-mice with a transgenic copy of *Sry* on an autosome (XY^- *Sry*, which are for all intents and purposes "males"), four types of offspring are obtained: XX (wild-type female), XX *Sry* (essentially, chromosomal females with testes), XY^- (essentially, chromosomal males with ovaries), and XY^- *Sry*.[157] Using this model, evidence has steadily accumulated for direct effects of sex chromosomes on the brain and behavior.

For example, midbrain neurons harvested from XY^- embryos show greater expression of tyrosine hydroxylase, the rate-limiting enzyme for dopamine biosynthesis, than do neurons harvested from XX embryos, regardless of gonadal sex.[158] The sex difference in vasopressin innervation of the lateral septum (see Table 47.1) is also in part dependent on sex chromosome complement, because animals with a Y chromosome (XY^- *Sry* and XY^-) have more vasopressin than XX mice, regardless of which gonads are present (Figure 47.6(A)).[122,157] Evidence has also been reported for direct, gonad-independent effects of sex chromosomes on aggressive and parenting behavior,[122] responses to noxious stimuli,[159] social and investigatory behaviors,[160,161] and habit formation.[162,163]

One caveat to the FCG model is that one can never be completely sure that XX *Sry* and XY^- *Sry* groups (XX and XY males) are exposed to exactly the same gonadal hormone levels perinatally. The testes of XX males are smaller than those of XY^- males and are infertile, for example, because genes on the Y chromosome other than *Sry* are required for spermatogenesis. It is possible

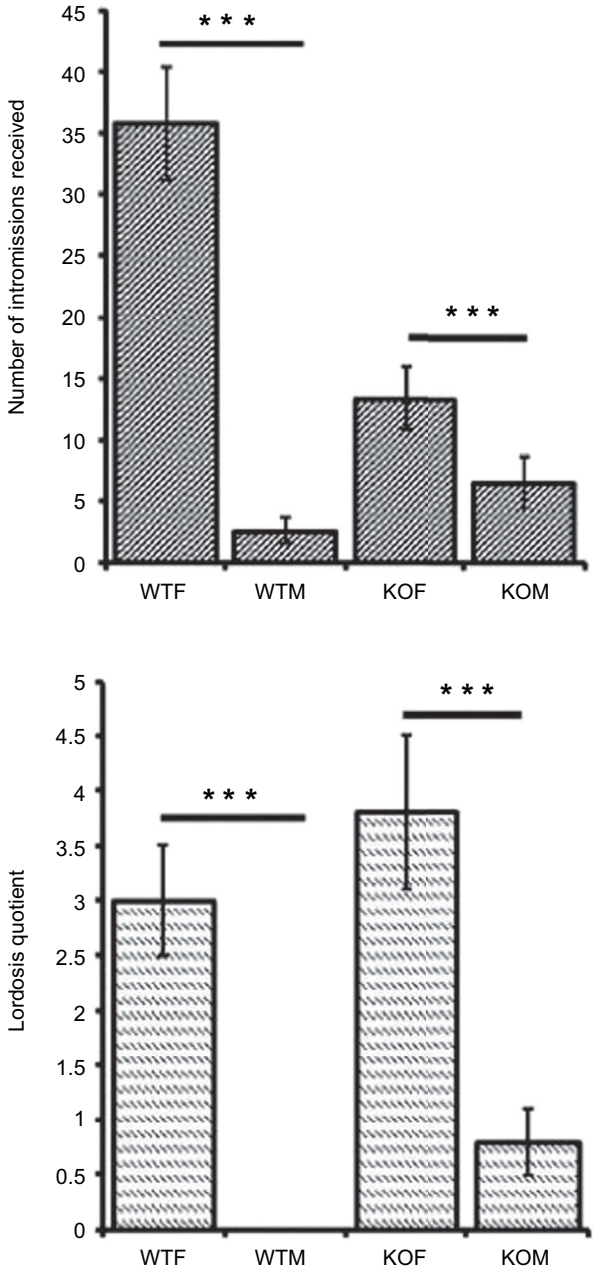

FIGURE 47.5 A sex difference in female sexual behavior persists in SF1-knockout mice. Wild-type female (WT F) and wild-type male (WT M) mice were compared to female and male SF1-knockout mice, which develop in the absence of gonads. Wild-type animals were gonadectomized at weaning, and all animals received estradiol and progesterone to stimulate sexual behavior. The top figure shows average lordosis quotient (a measure of receptivity), and the bottom shows intromissions received from the males over seven sexual behavior tests. Females show more feminine sexual behavior than do males, regardless of whether they are wild-type or SF1 knockouts. *Source: Adapted with permission of Elsevier from Ref. 155.*

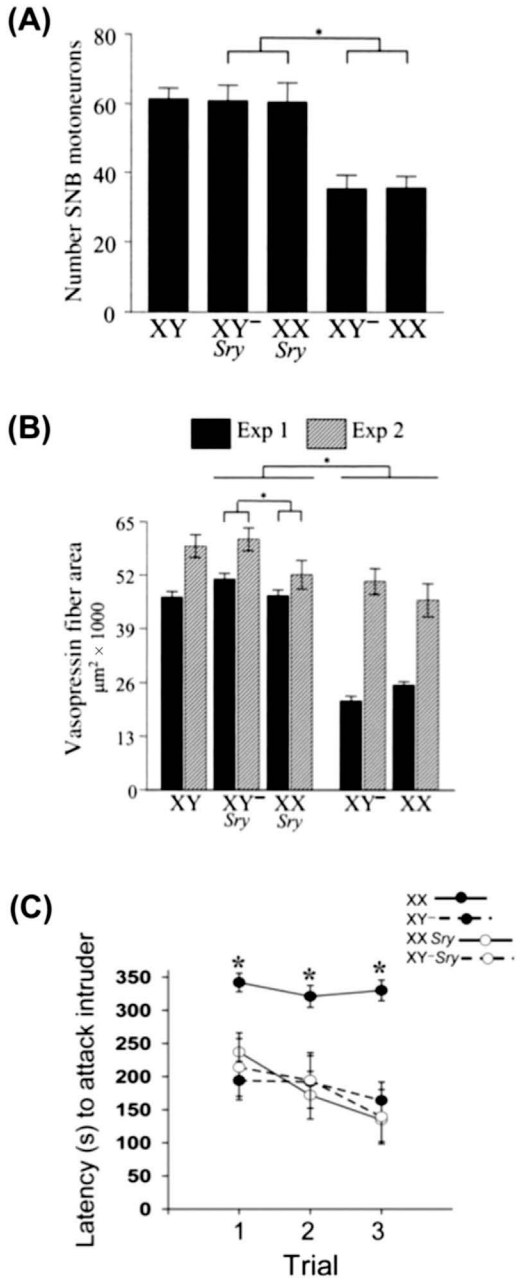

FIGURE 47.6 Four core genotype mice, in which genetic and gonadal sex are inherited independently, can be used to test for the effects of sex chromosome complement on sex differences in neuroanatomy, neurochemistry, and behavior. (A) The number of motoneurons in the spinal nucleus of the bulbocavernosus (SNB) was greater in gonadal male mice (XY⁻ *Sry* and XX *Sry*) than in gonadal females (XY⁻ and XX), with no effect of the sex chromosomes on this measure. (B) In contrast, there was a small but consistent effect of sex chromosomes on the density of vasopressin immunoreactive fibers in the lateral septum. As expected, gonadal males (XY⁻ *Sry* and XX *Sry*) had a higher density than did females (XY⁻ and XX), but in addition XY⁻ *Sry* males had a higher density than XX *Sry* males, showing an effect of sex chromosome complement. The same pattern of results was seen across two experimental replications (black bars and hatched bars). (C) Aggression latencies in resident–intruder tests over three consecutive days. All mice were adults, gonadectomized, and treated with a testosterone-filled implant. XX females were significantly slower to display aggression than all other groups. *Source: Adapted with the permission of the Society for Neuroscience from Ref. 127 (A and B), and Ref. 122 (C).*

that there are also subtle differences in hormone secretion by XX and XY testes. However, this worry is lessened by the observation that a number of traits known to be sensitive to circulating gonadal steroids are virtually identical in XX and XY males (e.g., the number of motoneurons in the spinal nucleus of the bulbocavernosus (SNB); Figure 47.6(B)).[157]

There are also some apparent inconsistencies between findings using the FCG and SF1-knockout models. For example, a resident-intruder paradigm has been used to examine whether there is a direct effect of sex chromosome complement on territorial aggressive behavior using both models. In both studies, all animals were tested in adulthood in the absence of gonads and with testosterone treatment. In the FCG study, XX mice were less aggressive than all other groups (XX *Sry*, XY⁻ *Sry*, or XY⁻) (Figure 47.6(C)).[122] This result suggests that either developmental exposure to testes (i.e., in those groups with the *Sry* transgene) or a Y chromosome in the absence of testes (XY⁻) is sufficient to confer a high level of aggression. However, using a similar behavioral test in the SF1 model, only wild-type males showed high levels of aggression.[164] XY mice that developed in the absence of gonads were no more aggressive than XX mice. An explanation may be found in the ways that the test was conducted in the two different laboratories (see the section Sexual Differentiation in Different Contexts) or differences in the endocrine "history" of the animals: SF1-knockout mice are exposed to steroids from the mother or from wild-type uterine neighbors, but not to secretions from their own gonads, whereas FCG mice are exposed to secretions from their own testes or ovaries in addition to maternal and littermate sources. A similar explanation may underlie the finding of a direct chromosome effect on vasopressin innervation of the septum in the FCG model,[122,157] but not in the SF1-knockout model.[156]

The gene or genes responsible for sex chromosome effects on behavior or neuroanatomy are not known. The most obvious candidates are Y chromosome genes, which are present only in males, or X chromosome genes, which may be present in different doses in males and females (Figure 47.7). All cells in female mammals possess two X chromosomes, while male cells possess only one. Early in development, one X chromosome is randomly inactivated in each female cell, more or less equalizing gene dosage.[165] However, X chromosome inactivation may not be complete and some genes on the X escape inactivation altogether.[166] These X-escapees could cause sex differences in gene expression and, hence, function. Some Y chromosome genes and X-escapees are in fact expressed in the brain.[167] For example, *Sry* itself is expressed in the brains of mice and humans.[168] Moreover, knockdown of *Sry* in dopaminergic midbrain neurons results in a decrease in expression of tyrosine hydroxylase (the rate-limiting enzyme in dopamine production) and impaired motor function, but only in males.[169] Recent evidence

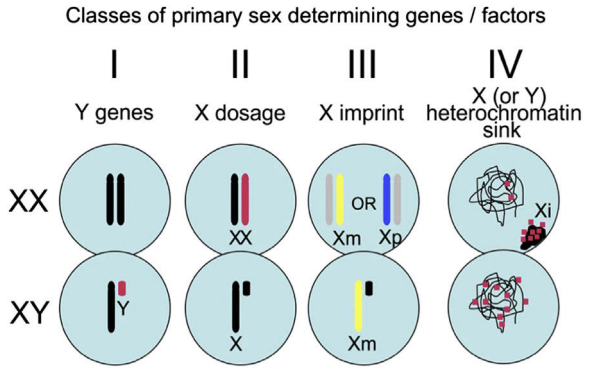

FIGURE 47.7 Four ways that sex chromosomes could affect gene expression. This figure is reproduced in color in the color plate section. Four possible classes of primary sex-determining factors are recognized. Class I comprises Y genes that have a male-specific effect on one or more tissues. Class II is X genes that are expressed at a higher level in females than males by virtue of the 2:1 ratio in the number of X chromosomes. Class III is X genes that receive a parental imprint. The X chromosome receiving a maternal imprint (Xm, yellow) is active in half of XX cells and all of XY cells, whereas the X chromosome receiving a paternal imprint (Xp, blue) is active in half of XX cells and no XY cells. Class IV comprises proposed regions of the sex chromosome heterochromatin (the heterochromatic inactive X (Xi) is illustrated here) that act as sex-specific sinks for factors (red dots) that regulate the amount of euchromatin–heterochromatin at interphase and therefore affect gene expression throughout the genome. To date, specific members of only Class I have been identified (*Sry* and spermatogenesis genes). Although evidence indicates that the number of X chromosomes leads to some sex differences in phenotype, the specific genes or chromosome regions that explain these X effects have not been identified. Class IV is particularly speculative at present because it is based on a limited number of studies. Future studies are likely to expand the importance of Classes II–IV. *Source: Adapted with permission of Elsevier from Ref. 58.*

suggests that *Sry* similarly regulates tyrosine hydroxylase expression in dopaminergic neurons of humans.[170] Note that differences in *Sry* would not explain the direct effects of the sex chromosomes in the FCG model because *Sry* is present (or absent) in both comparisons (XY^- versus XX, and XY^- *Sry* versus XX *Sry*).

In addition to X and Y gene dosage, there are at least two other ways in which sex chromosome complement causes male and female cells to differ (Figure 47.7). One is a difference in parental imprints (essentially, epigenetic marks that affect gene expression). Only females receive an X chromosome from their fathers, so only females have a paternally imprinted X. In addition, recent evidence suggests that the X chromosome could act as a heterochromatin "sink". The inactivation of one entire X chromosome in each cell of XX mammals presumably requires a significant amount of epigenetic machinery to maintain. This large area of heterochromatin may also interact with other parts of the genome, an idea that receives direct support from observations in fruit flies[171] and may also explain some sex differences in gene expression in mammals.[58,172]

It is also important to keep in mind that expression of X or Y genes underlying gonad-independent effects on the brain and behavior could be peripheral (i.e., outside of the brain). In this scenario, signals from the periphery stemming from a sex difference in X or Y genes then change gene expression in the brain. Recent work has demonstrated sex differences in gene expression in, for example, the liver, adipose tissue, and muscle,[173] some of which is independent of the gonads.[174] Although rarely considered, this type of indirect effect of sex chromosome genes on the brain would, in fact, be analogous to the very familiar actions of Y chromosome on the brain via its role in directing differentiation of the developing gonads (see the section What Will the Future Bring? for additional consideration of this idea).

EFFECTS OF GONADAL HORMONES: CELLULAR AND MOLECULAR MECHANISMS

In principle, sexual differentiation of any neural trait must involve one (or a combination) of four basic cellular mechanisms: cell birth, cell migration, cell death, and the differentiation of neuronal phenotype. It is often difficult to identify which of these mechanisms contributes to a sex difference of interest. For example, assume that in a certain brain region, males have more neurons than do females that express neurotransmitter "x". If this sex difference persists even after adult hormone levels are made equivalent, but can be reversed by developmental manipulation of gonadal steroids, we would say that this difference is programmed by gonadal steroids. This difference, however, could have arisen because steroid hormones caused more neurons destined for the region in question to be born in males (i.e., a hormonal effect on neurogenesis), or because steroids affected the movement and aggregation of cells, such that more cells overall came to be associated with the brain region of interest in males. The difference also could have arisen because fewer cells died in males (an effect on cell death), or because gonadal steroids caused existing cells to express neurotransmitter "x" (i.e., a hormonal effect on neurochemical differentiation). Of these four mechanisms of sexual differentiation, evidence is best for cell death, followed closely by phenotypic differentiation. The extent to which differential cell birth or migration contributes to known neural sex differences is less clear.

Neurogenesis

Neurogenesis often can be ruled out as a factor, because for some neural sex differences, all cells are born before the gonads differentiate. For example, motoneurons that populate the SNB undergo their final mitosis

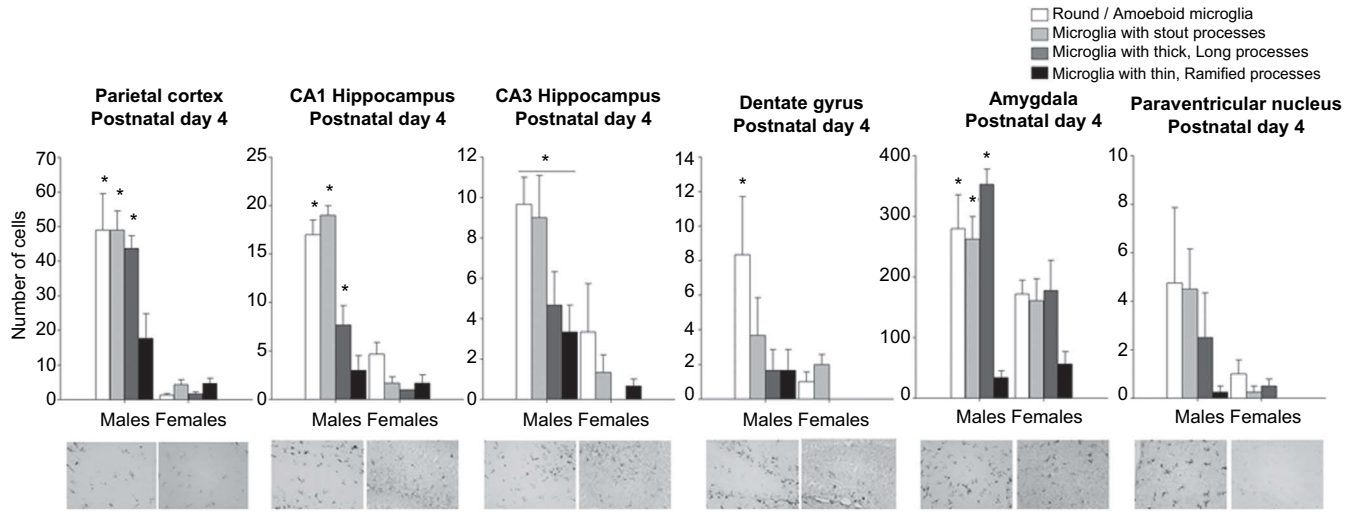

FIGURE 47.8 The morphology and number of microglia are affected by sex in the postnatal day 4 rat brain. Microglia were identified using immunohistochemistry for ionized calcium binding adapter molecule 1 (Iba1). All brain regions displayed more amoeboid microglia and microglia with stout processes than microglia with thicker or thinner processes at this age. Within the parietal cortex, the CA1 and CA3 regions of the hippocampus, the dentate gyrus, and the amygdala, there was a significant interaction of sex and microglial morphology (within-subjects ANOVA, $p < 0.05$; $n = 4$ rats/group). Males had more amoeboid microglia, microglia with stout processes, and microglia within thick, long processes than females within the parietal cortex, CA1 hippocampus, and amygdala. Within the CA3 of the hippocampus, males had significantly more microglia in all morphological categories than females; and within the dentate gyrus of the hippocampus, males had significantly more amoeboid microglia than females. No effects of sex on glial morphology or number were detected within the paraventricular nucleus. Data represent the mean ± SEM of all Iba1+ cells in each morphological category across all sections analyzed. *Source: Reprinted with permission of John Wiley and Sons from Ref. 182.*

by the 14th day of gestation in rats,[175] several days before testosterone will be produced by the testes. In other cases, however, gonadal steroid production and cell birth overlap. This is true for the SDN-POA, for example.[68,176] Using tritiated thymidine or bromodeoxyuridine to label dividing cells and examining the number of cells labeled after very short survival times (to rule out effects due to death or migration), neurogenesis has been eliminated as important for the initial differentiation of the SDN-POA.[177] Interestingly, neurogenesis could still contribute to the maintenance of this sex difference.[178] In brain regions where neuron production continues throughout life, hormones could create sex differences in cell number by controlling this cell production. For example, estradiol increases neurogenesis in the dentate gyrus of female rats.[179] However, this does not result in differences in total cell number in this area, presumably because increases in cell death are offset by increases in cell birth. At present, no sex difference has been convincingly linked to the hormonal control of neurogenesis, although this may be a possibility in the developing CA1 region of the hippocampus.[180,181]

Although this section is titled "Neurogenesis", at least half of the cells in any given brain region are glial cells, and sex differences in glial cell number have been described (Figure 47.8).[114,182–185] Thus, the hormonal control of gliogenesis may also be an important mechanism for sexual differentiation, and this is clearly an area deserving more attention. Because glial cells are born throughout life, hormonal effects on gliogenesis could in theory happen at any time, including the pubertal period. For example, the number of astrocytes in the rat medial amygdala is equal in the two sexes prepubertally, thereafter increasing much more in males than females. The greater increase in males is hormonally driven, because genetic males with defective androgen receptors show a more modest, female-typical increase in astrocytes by adulthood.[116] Sex differences in the number of microglia are also seen in several brain regions during early postnatal life,[182] although whether this is due to differences in the genesis of microglial cells or to some other mechanism (e.g., migration) is not yet known.

Cell Migration

Like neurogenesis, cell migration can be excluded as an important factor for many of the well-known sex differences in the brains of mice and rats (e.g., in the POA, AVPV, and principal nucleus of the bed nucleus of the stria terminalis (BNSTp)), as most cells in these regions have migrated to their respective location prior to the perinatal sex difference in testosterone synthesis.[186–188] However, in some brain regions, cells are added throughout perinatal development,[109,186] and even for cells that are already in place before testosterone levels surge in males, gonadal steroids may cause subtle changes in cell position within a general brain region. For example, adult male and female rats have similar numbers of ERβ-expressing cells in the AVPV, but these cells are more medially located in females.[189] This sex difference

in the position of cells, however, is not necessarily due to sex differences in migration. For example, if testosterone increases the survival of cells in the lateral but not the medial AVPV, then males will wind up with more laterally positioned cells.

To address the question of whether cell migration plays a role in sexual differentiation, Stuart Tobet et al. followed the migration of tagged cells in organotypic cultures of the POA–anterior hypothalamus (AH) of embryonic mice. They noted that males showed significantly more medial-to-lateral migration than did females,[190] which matched well with a transient sex difference in the location of phenotypically identified cells in the POA–AH two days later.[191] Moreover, they showed that this migration was affected by estradiol, but not by the androgen dihydrotestosterone, which cannot be converted to an estrogen,[192] and that GABA receptor antagonists influenced cell migration as well.[193] This latter observation suggests that sex differences in neural activity could influence cell positioning.

Although the functional significance of sex differences in cell migration is not clear, one could imagine that the positioning of cells might affect afferent input, cell-to-cell communication on a local scale, or exposure to diffusible signals. For example, the closer proximity of ERβ-expressing cells in the AVPV to the ventricle in females could increase access to chemosignals in the cerebrospinal fluid.

Cell Death

Development of the nervous system is characterized by a period of developmental cell death, or apoptosis, during which about half of the neurons initially produced are thought to die,[194] and gonadal steroids can modulate this cell death. Of all the cellular mechanisms considered as factors in sexual differentiation of the brain, differential rates of developmental cell death are currently the best established. Nonetheless, only in a handful of cases is the evidence rather complete.[177] These include some of the most "famous" sex differences, including the SNB, SDN-POA, BNSTp, and AVPV in rats.[68,69,87,101] Cell death may also play a role in sexual differentiation of the rat visual cortex,[195] the gerbil preoptic area,[196] at least one birdsong nucleus,[197] and frog laryngeal motoneurons.[198] In each of these cases, sex differences in cell numbers in adulthood are correlated with sex differences in the number of dying cells at some point in development. For example, adult males have more SNB motoneurons than do females, and females have more pyknotic cells in the SNB region around birth.[87] Early postnatally, females also have more pyknotic cells than do males in the BNSTp,[69,109] which correlates with greater BNSTp cell number in adult males. In both cases, developmental testosterone treatments that eliminate the sex difference in size and cell number of these nuclei in adulthood also eliminate the sex difference in apoptosis during development,[69,87] supporting the conclusion that differential cell death is the mechanism underlying sexual differentiation in these cases.

Counting pyknotic or TUNEL-positive cells to determine the rate of cell death has its limitations because such cells provide merely "snapshots" of dying cells at a particular stage of apoptosis. For example, the rat locus coeruleus shows more neurons in adult females than in males, which corresponds with greater rates of cell death in males at birth,[199] suggesting that differential rates of cell death cause this difference. However, a more fine-grained developmental study of the changes in cell number suggests that the adult sex difference may actually result from the addition of cells in the female locus coeruleus after puberty.[200] A similarly complex situation applies to the greater number of cells in the visual cortex of males versus females.[201]

Recently, mice "mutation for cell death" genes have been used to get beyond correlational studies and to test more directly the contribution of differential cell death to sexual differentiation of the brain. The period of developmental neuronal cell death occurs perinatally in mice, and cell death falls to very low levels in most brain regions after the first week of life.[202] In mice carrying a deletion in *Bax*, a pro-death gene of the Bcl2 family, this neuronal cell death is virtually eliminated.[202,203] In these mice, the number of cells in the SNB, AVPV, and BNSTp is significantly increased,[88,105] and, more importantly, sex differences in the total cell number in each of these regions are completely eliminated (Figure 47.9). Thus, a functional *Bax* gene is required for sexually dimorphic cell death in the mouse forebrain and spinal cord.

Besides allowing us to identify genes necessary for sexually dimorphic cell death, cell death mutants also allow us to estimate more precisely the contribution of cell death to a given neural sex difference. Although not all neural regions require Bax protein for cell death, for many regions Bax is essential for this process.[202,203] If this is true for the SNB, AVPV, and BNSTp as well, then the difference in neuron number between Bax−/− and Bax+/+ mice represents the total number of neurons lost to cell death, integrated over the entire developmental cell death period. In the AVPV, BNSTp, and SNB, the cell number is equivalent in male and female Bax−/− mice,[88,105] suggesting that cell death alone can account for the sex difference in the overall cell number in these regions in wild-type animals. If the numbers were different, other mechanisms in addition to cell death would have to be considered. In the same vein, studying Bax−/− mice allows us to identify sex differences not dependent on Bax, and demonstrates that the control of cell number may vary not only from region to region, but also among

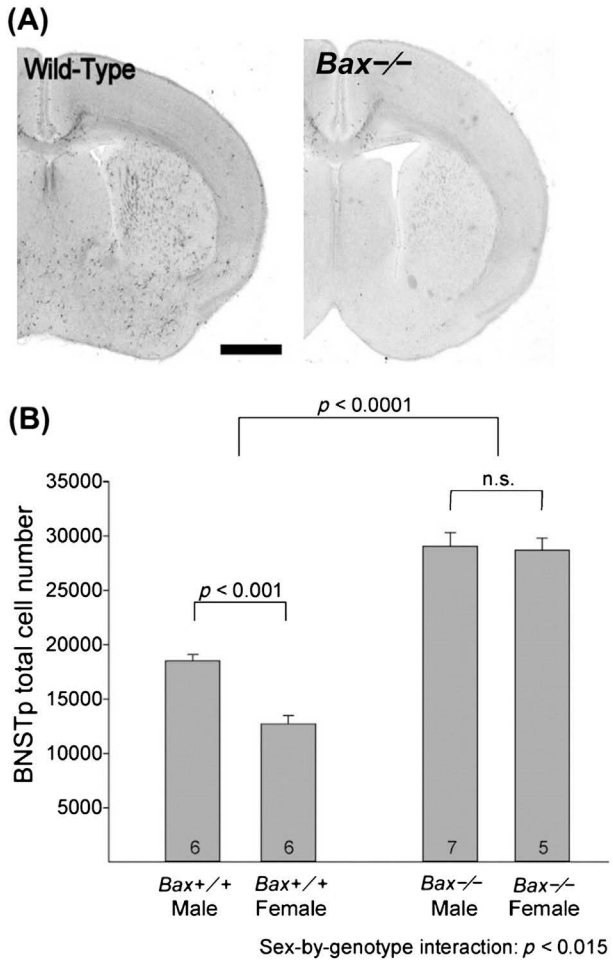

FIGURE 47.9 The effect of preventing developmental cell death on a sex difference in the principle nucleus of the bed nucleus of the stria terminalis (BNSTp). (A) Cell death throughout the developing mouse brain is dependent on the pro-death gene, *Bax*. Dying cells (black dots), labeled by immunohistochemistry for activated caspase 3, are much more numerous in newborn wild-type mice (*Bax*+/+) than in Bax-knockout mice (*Bax*−/−). (B) In adulthood, cell number in the BNSTp was greater in wild-type males than in females. Deletion of the *Bax* gene increased cell number overall and eliminated the sex difference, suggesting this sex difference depends on developmental cell death. The number of animals per group is indicated at the base of each bar. n.s.: not significant. *Source: Reprinted with permission of Wiley from Ref. 202 (A), and reprinted with the permission of the National Academy of Sciences of the USA from Ref. 105 (B).*

subtypes of cells within a single region (Refs 105,106,123; and discussed further in this chapter).

A central question is how gonadal steroids such as testosterone regulate cell death. Many studies have linked the steroid hormone-regulated death of peripheral tissues and cancer cells to changes in the ratio of pro-life and pro-death Bcl2 family members. Steroid hormones also regulate the expression of Bcl2 family members in neural tissue.[177] This regulation may be direct in some cases, since the Bcl2 and BclxL genes contain putative estrogen response elements.[204,205] Because research on steroid effects on Bcl2 family members has been primarily geared toward understanding the neuroprotective effects of estrogens in injury models, we do not know much about the hormonal control of Bcl2 family proteins during development. Interestingly, however, testosterone as well as estradiol regulates Bcl2 and Bax expression in the SDN-POA of newborn rats.[206,207] Thus, the mechanisms by which estradiol regulates cell survival in adult peripheral tissues may also apply to sexual differentiation of the brain.

An interesting new line of research suggests that sexual differentiation of specific brain areas and behaviors uses components of signal transduction pathways that are common to inflammatory processes.[208–210] Some of these components may be involved in cell death as well. For example, using microarrays, Krishnan et al. demonstrated that genes involved in the tumor necrosis factor α (TNFα)-TNF receptor 2 (TNFR2)-NF kappa B cell survival pathway are expressed at higher levels in the female AVPV than in the male AVPV on postnatal day 2.[102] The sex differences illustrated here in the number of microglia in various brain regions of the postnatal rat may provide another example, as these are the resident immune cells of the brain. The rat preoptic area provides a case in point. At postnatal day 2, males have twice the number of activated microglia as females, and microglial inhibition at that time prevented sex differences in dendritic spine density and adult masculine sexual behavior (Figure 47.8).[66] Immune–nervous system interactions may also help to explain the sexually dimorphic effects that infections during development have on brain development.[211]

Differentiation of Neural Morphology and Neurochemistry

Newly generated neurons that have migrated and survived the cell death period differentiate into myriad forms that differ in size, shape, dendritic and axonal ramification, the number and location of synapses, the palette of neurotransmitters expressed, and the types of receptors that adorn their membranes or reside intracellularly. Each of these features may affect the function of neuronal circuits and can be altered by gonadal steroids. Rather than exhaustively reviewing the literature here, we present a number of salient examples of sex differences in, or gonadal steroid hormone regulation of, morphological (axon outgrowth, dendritic branching, and synaptogenesis) and neurochemical differentiation.

Axon Outgrowth

A number of sex differences in axonal projections from one brain region to another have been described. It is not so easy to determine, however, whether these are due to sex differences in axonal outgrowth per se or to some other process (e.g., a sex difference in neurogenesis or cell

death that alters the number of cells contributing to the projection). Projections to and from the AVPV provide examples where this issue has been addressed. Unlike most of the sexually dimorphic regions studied to date, the AVPV is larger and more cell dense in *female* rats and mice than in males (see Table 47.1). Anterograde labeling experiments indicate that neurons in the AVPV provide direct inputs to gonadotropin-releasing hormone (GnRH)-containing neurons in the preoptic region[212] (see also Chapter 11). Many of the neurons projecting to GnRH cells are kisspeptin-expressing cells that are much more numerous in females,[213,214] and this sexually dimorphic input may explain why male rodents have relatively tonic luteinizing hormone (LH) release, while females exhibit cyclical LH surges. Similarly, descending projections from the AVPV to dopaminergic neurons in the arcuate nucleus are more abundant in female rats than in males.[215] Although these are bonafide examples of sex differences in axonal projections, it is not currently known whether these sex differences are due to differences in axon outgrowth or to an alternative mechanism.

Somewhat more direct evidence comes from considering the projection from the BNST to the AVPV, which is about 10 times denser in male rats than in females. Developmental studies suggest that a massive projection from the BNST to the AVPV is established between postnatal days 9 and 10 in males, but never forms in females.[216] This is apparently due to hormone action at the target tissue (in this case, AVPV), as determined by mix-and-match co-culture experiments (Figure 47.10). When explants of the BNST and AVPV taken from neonatal male or female rats were co-cultured in the same dish, robust outgrowth from the BNST to the AVPV formed only if the AVPV came from a male or a testosterone-treated female.[217] AVPV explants from females did not support outgrowth from either male or female BNST explants. This result suggests that either an unknown diffusible factor from the AVPV of females repels BNST axons, or a factor from the AVPV of males (or testosterone-treated females) attracts BNST axons.

Hormone effects on axon outgrowth no doubt occur in other brain regions as well and may not always involve hormone action at the target site. For example, estradiol increased axon elongation of rat embryonic hypothalamic neurons grown in culture,[218] which suggests a direct effects of steroids on the cells growing axons, unless the target cells were also present in the culture. Speert et al.[219] recently provided a potential molecular explanation for the hormonal regulation of axonal outgrowth. They found that the hypothalamus of neonatal female rats has higher levels of focal adhesion kinase (FAK) and paxillin, two molecules associated with cell adhesion and axon growth, than that of males. These sex differences were reversed by treating female neonates with estradiol or by administering an aromatase inhibitor to the males, indicating that they are due to the perinatal gonadal hormone milieu. Because FAK and paxillin are involved in axon outgrowth, sex differences in these molecules, or others like them, could underlie regional sex differences in outgrowth.

Dendritic Growth and Branching

The earliest work pertaining to the hormonal control of dendritic growth came from in vitro studies by Toran-Allerand, who showed that estradiol greatly increased the neurite outgrowth from organotypic explant cultures of the newborn mouse hypothalamus and preoptic area.[220] The term "neurite" was used in the initial studies because any given outgrowth from an explant could be an axon or dendrite (or, for that matter, a glial protrusion[221]). Based on Golgi staining of cultured neurons, however, at least some of the effect is due to estrogen action on dendrites.[222] Immunocytochemical markers can specifically label dendrites in explant studies, or, alternatively, dendritic identity can be inferred based on cell morphology in cultures of dissociated neurons.

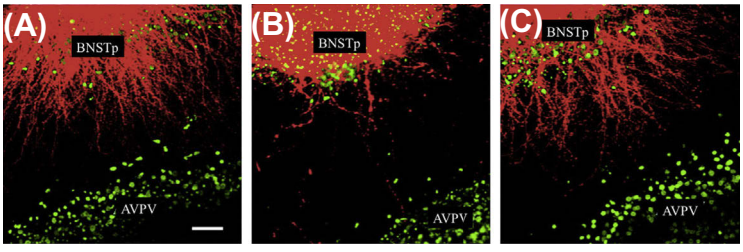

FIGURE 47.10 Target-dependent induction of neurite outgrowth in co-cultures of the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) and anteroventral periventricular nucleus (AVPV). This figure is reproduced in color in the color plate section. Confocal images of BNSTp-AVPV co-cultures show DiI labeling of neuritis (pseudo-colored red) that extend from the BSTp toward the AVPV. The BSTp explant was derived from a male rat on postnatal day 5 (PN5) and co-cultured with an AVPV explant (pseudo-colored green) derived from a PN9 (A) male, (B) female, or (C) androgenized female. A marked difference in the density of neurites between the co-cultures explants was observed, with many more neuritis seen when the AVPV explant was derived from a male (A) versus a female (B) rat. Treatment of neonatal female rats with testosterone during the first 9 days of life in vivo masculinized (i.e., increased) neurite outgrowth from BSTp explants (C), suggesting that the target-dependent formation of neuritis extending from the BSTp to AVPV is determined by exposure to sex steroid hormones during the neonatal period. Scale bar: 15 μm. *Source: Adapted with the permission from Ref. 217.*

Using these approaches, estradiol increased dendritic branching in cultures of embryonic rat medial amygdala.[223] Effects of estradiol are not always stimulatory, however: estradiol inhibited neurite growth of serotonergic neurons of embryonic rats in culture.[224]

Unfortunately, studying dendritic outgrowth in the actual brain turns out to be quite challenging, and evidence for sex differences in dendritic trees has been sparse. Some of the earliest evidence for an effect of gonadal steroids on dendrites came from studies on songbirds. Dendritic extent is much greater in male canaries, which sing, than in females, which don't sing, within the song control nucleus known as RA. De Voogd and Nottebohm found that testosterone treatment of female canaries in adulthood increased dendritic lengths to male-like levels.[225] Similar effects can be seen in mammals: castration of adult male rats reduced the dendritic extent of motoneurons in the SNB (see Table 47.1) by 50%, and this could be prevented by treating castrates with testosterone.[226] Shrinkage of SNB dendrites was also seen in male white-footed mice exposed to short, winter-like day lengths,[227] suggesting that hormonally mediated growth and retraction of SNB dendrites may be normal features in seasonally breeding rodents. Gonadal hormones also control the initial outgrowth of SNB dendrites during postnatal development; in this case, however, both estrogens and androgens are implicated.[228]

The SNB may also be the only neural region in which the site of hormone action for effect on dendritic trees has been examined. Interestingly, testosterone seems to act not on the SNB motoneurons themselves, but at the target muscles to control SNB motoneuron dendritic extent in adulthood.[229] Similarly, in development, the effect of estrogens on SNB dendrites appears to be mediated by hormone action at the target muscles. Administering estrogens at the target muscle supports the dendritic growth of developing SNB motoneurons, and blocking ERs at the muscles results in stunted dendritic trees.[230]

Few studies have directly examined sex differences or effects of gonadal hormones on dendritic trees in the mammalian brain, which is likely related to the fact that the techniques required can be capricious and are very time consuming. Motoneurons such as SNB cells can be labeled relatively easily by injecting retrogradely transported tracers into their target muscles, but much more tedious Golgi impregnation studies or direct dye filling are generally required in the brain. Based on Golgi staining, sex differences in dendritic trees were first described in the preoptic area of hamsters.[231] Also using the Golgi technique, dendrites in the adult ventromedial nucleus of the hypothalamus (VMN) were found to be longer in male rats than females,[232] a somewhat surprising result since the VMN is a major site for the hormonal control of female copulatory behavior. On the other hand, the regional volume of the VMN is greater in male rats than in females, a sex difference that is dependent upon functional androgen receptors.[233] and there is good evidence that this nucleus plays a role in aggressive behavior in mice.[234] Estradiol and progesterone have opposing effects on dendrite length in the VMN in females,[235] and, in fact, dendritic length in the VMN can change from day to day over the course of the estrous cycle.[236] This suggests rapid, hormone-dependent plasticity in the circuitry underlying female sexual behavior.

Most other evidence pertaining to sex differences and hormonal effects on dendrites has focused on dendritic spines, protuberances on dendrites that are often the sites of excitatory synapses, or by quantifying synapses themselves.[64,231,237–243] In the arcuate nucleus of the hypothalamus, for example, females have a two- to threefold higher density of axodendritic spine synapses than males, while males have more axosomatic synapses than do females. This sex difference is reversed in males castrated as neonates or females treated with testosterone at birth.[239] Because axosomatic synapses are generally inhibitory while axodendritic spine synapses are excitatory, a sex difference in the relative number of somatic versus spine synapses is likely to affect neuronal excitability.

In a few cases, researchers have made headway in identifying the mechanisms underlying the sexual differentiation of dendritic spines. For example, male rats have a much greater density of dendritic spines in the POA than do females.[64] The induction of spines in the POA of males is established in the first few days of postnatal life, is due to estradiol, and appears to be permanent (Figure 47.11).[208] The molecular mechanism involves an estrogen-dependent increase in the enzyme cyclooxygenase-2 (COX-2) and its product, prostaglandin E2. Increased prostaglandin E2 in the neonatal POA is then responsible for increased spine formation in males.[244] The molecular basis of hormone effects on dendritic spines is regionally specific, however. In the VMN, for example, the neurons in male rats have longer dendrites with more excitatory dendritic spine synapses than in females.[244] In this case, estradiol acts not via prostaglandins, but by increasing glutamate release from presynaptic neurons. This glutamate acts via ionotrophic glutamate receptors on the postsynaptic cell to induce spine formation.[243]

Although the foregoing examples suggest a programming effect of hormones early in life, in some cases dendrites (and/or their spines) remain sensitive to changes in gonadal steroids in adulthood. In addition to the effects of gonadal steroids on dendritic trees in adult songbirds and the SNB, mentioned in this chapter, dendrites and dendritic spines may be influenced by circulating levels of steroids in the peripubertal (e.g., Ref. 240) or adult mammalian brain (e.g., Refs 245,246). Dendritic spine density can also change quite rapidly, for example from day to day over the estrous cycle.[236,247]

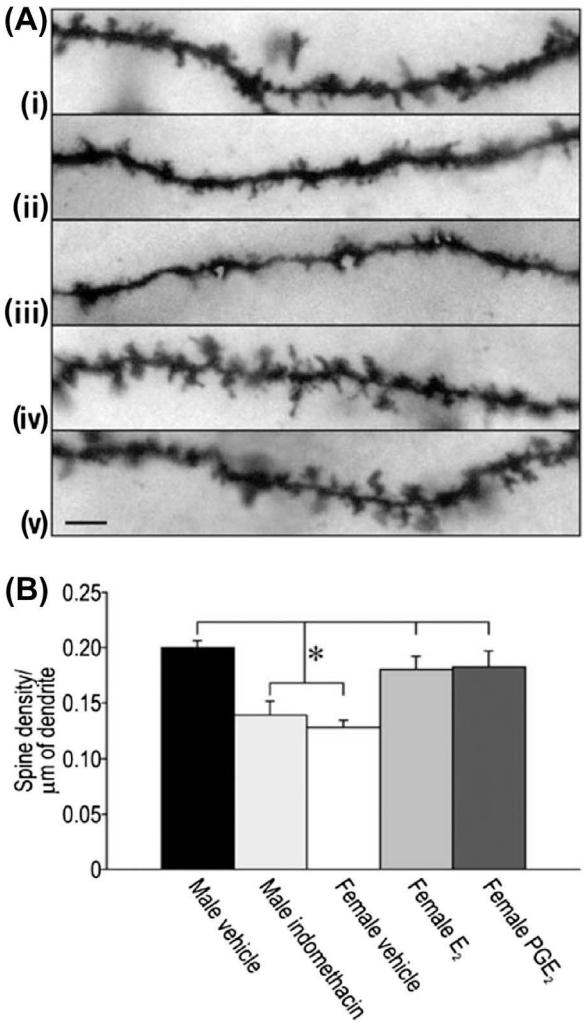

FIGURE 47.11 Estradiol and prostaglandin E_2 (PGE_2) increase dendritic spine density in the developing preoptic area (POA) of the rat. (A) Photomicrographs of Golgi–Cox impregnated dendrites extending from male POA neurons on postnatal day (PN) 25 after 48 h of neonatal exposure to (i) vehicle, or (ii) 25 µg indomethacin (an inhibitor of prostaglandin synthesis), and those from female POA neurons exposed to (iii) vehicle, (iv) 100 µg estradiol (E_2), or (v) 2 µg PGE_2. (B) Quantification of the spine densities from these five treatment groups showing significant treatment effects indicated by asterisk. *Source: Used with the permission of Nature Publishing Group from Ref. 208.*

Neurochemical Phenotype

Neurochemical phenotype in its broadest sense refers to all of the chemicals stably expressed in a given neuron. For illustrative purposes, we restrict our discussion here to the expression of neurotransmitters, which show numerous sex differences.[248–250] Given any sex difference in neurotransmitter expression in a brain region, two fundamentally different sets of processes could be involved: processes that determine the absolute number of cells capable of expressing a specific transmitter (i.e., birth, death, or migration of cells) or processes that act on preexisting cells to direct their transmitter expression. It can be surprisingly difficult to disentangle these two possibilities, in part because gonadal hormones often trigger sexual differentiation before the neurons of interest assume their final phenotype.

A case in point is the sexually dimorphic vasopressin innervation of the brain (Table 47.1). This innervation shows one of the most consistently found neural sex differences among vertebrates,[133] with males having more vasopressin neurons than females in the BNST and medial amygdala, and denser projections from these areas, across many mammalian species (Figure 47.12).[133] Nonmammalian vertebrates show similar sex differences in homologous vasotocin projections.[133,134,252] This sex difference has been well studied in rats, where exposure to gonadal steroids during perinatal life determines the number of vasopressin cells found in adults.[124,125] In theory, hormones might influence cell birth, migration, cell death, or cellular differentiation to alter the number of vasopressinergic cells. Differential cell birth and migration are very unlikely to contribute in this case, because vasopressin cells are born on embryonic days 12 and 13,[188,253] prior to differentiation of the gonads and at least a week before gonadal hormones trigger their sexual differentiation. This leaves differential cell death or phenotypic differentiation as the two most likely causes.

Early work provided some circumstantial evidence favoring the differentiation of phenotype. Essentially all vasopressin cells in the BNST co-express the neuropeptide galanin, but not all galanin cells co-express vasopressin.[254] Because the total number of galanin cells does not differ between males and females,[255] it was hypothesized that, during development, higher levels of testosterone act on existing galaninergic cells to increase the percentage that will co-express vasopressin.[254] This hypothesis is difficult to test directly. Although gonadal steroids determine vasopressin cell number soon after birth, the vast majority of presumptive vasopressin neurons do not begin expressing the peptide until several days (in males) to weeks (in females) later.[256] Thus, one cannot identify the cells of interest during the time the sex difference in their number is determined.

Instead, efforts turned to assessing whether cell death could be eliminated as a factor contributing to the sex difference. To do this, vasopressin cell number was examined in the BNST of mice overexpressing the pro-survival protein, Bcl2, or mice lacking the pro-death protein, Bax. Both Bcl2 overexpressors and Bax knockout mice have greatly reduced neuronal cell death, so if cell death is required for the sex difference in vasopressin cell number, it should be eliminated in these animals. These mutations did indeed increase the total number of cells that produce vasopressin in both sexes. Critically, however, the sex difference in cell number remains intact.[123] That is, cell death determines the number of cells with

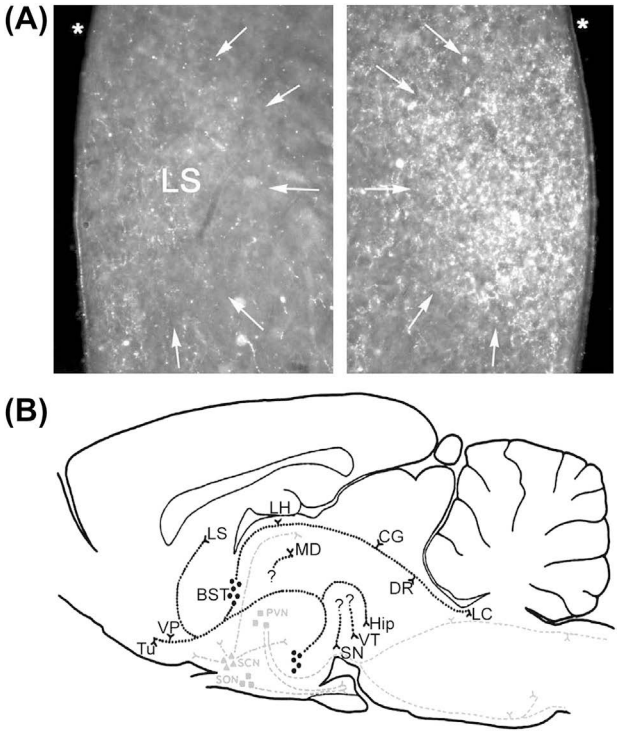

FIGURE 47.12 (A) AVP-immunoreactive fibers (arrows) in the lateral septum (LS) of an intact female (left) and male rat (right). *: Lateral ventricle. (B) Schematic diagram depicting sites of vasopressin synthesis in the rat brain. A limited number of cell groups produce vasopressin (dark circles and gray squares and triangles). The paraventricular nucleus (PVN) and supraoptic nucleus (SON) contain mangnocellular vasopressin neurons that project into the posterior pituitary gland via the median eminence. These cells release vasopressin into the bloodstream to control peripheral tissues. In addition, the suprachiasmatic nucleus (SCN), BNST, and medial amygdala (MeA) contain parvocellular vasopressin cells that project centrally. Vasopressin released from these sites controls circadian rhythms, social behaviors, and other functions. The BNST and MeA projections are sexually dimorphic, with more cell bodies and denser projections in males. Other abbreviations: VP: ventral pallidum; Tu: olfactory tubercle; LH: lateral habenular nucleus; CG: midbrain central gray; DR: dorsal raphe nucleus; LC: locus coeruleus; Hip: ventral hippocampus (Hip). Question marks indicate projections to the mediodorsal nucleus of the thalamus (MD), ventral tegmental area (VT), and substantia nigra (SN), all of which disappear after castration but not after lesioning the BNST. Relatively steroid-insensitive projections (gray lines) originate in the SCN (triangles), PVN (squares), and supraoptic nucleus (SON: squares). *Source: Reprinted with permission from Elsevier from Ref. 251.*

the potential to become vasopressinergic, but does not direct the sex difference. By elimination, this leaves sexual differentiation of cellular phenotype as the only remaining plausible mechanism for sexual differentiation of vasopressin expression.

Numerous other neurotransmitters and neuropeptides (e.g., dopamine, neurotensin, substance P, and enkephalin) show sex differences in cell number (reviewed in Ref. 248), and for none of these has the cellular mechanism of sexual differentiation been established. The search for these mechanisms may be amenable to the same strategies described for vasopressin. If, however, one successfully eliminates cell birth, migration, and death as possible mechanisms, that is, in some respects, only a modest first step. One then faces the task of identifying the molecular mechanism contributing to the phenotype of differentiating neurons.

How might hormones direct the phenotypic differentiation of a neuron? The most likely answer is epigenetics, the same mechanism responsible for the differentiation of cell type throughout the body of a developing embryo. In the case of the sex difference in vasopressin cell number, this might involve, for example, the placement of long-lasting epigenetic marks on the promoter region of the vasopressin gene that ensure expression of the gene in males, or inhibit it in females. Recently, Forbes-Lorman et al. used small interfering RNAs (siRNAs) to transiently reduce expression of the DNA methyl binding protein, methyl CpG binding protein 2 (MeCP2), in the amygdala of male and female rats during neonatal life. A transient reduction in MeCP2 eliminated the sex difference in vasopressin expression in the medial amygdala and BNST of rats.[257] This is consistent with a critical role for DNA methylation in sexual differentiation of the vasopressin system, although it does not address whether the change in DNA methylation is on the vasopressin gene itself or on some other gene that then influences vasopressin expression.

Role of Epigenetic Factors

As we have seen, the effects of gonadal steroid hormones can be permanent or long-lasting and are often characterized by a delay. As mentioned in this chapter, testosterone levels present neonatally determine the number of vasopressin neurons that project to the lateral septum, but the expression of vasopressin by those neurons does not begin until days (in males) to weeks (in females) later.[118,125,256] Presumably, there is a cellular "memory" for the testosterone exposure that gets revealed when the cell reaches a certain developmental stage. Similarly, estrogenic metabolites of testosterone administered on the day of birth reduce cell death in the BNSTp, but that cell death does not occur until nearly a week later,[69,109,202] and effects of neonatal testosterone on AVPV volume are not apparent until long after the hormone is cleared from circulation.[258] Some effects of perinatal steroids may not be observed until adulthood. The basis for such cellular memory has been mysterious until recently, but an explanation may be found in the rapidly exploding field of neuroepigenetics.

Although the definition of epigenetics is not widely agreed on and is still evolving, we use it here to refer to alterations in chromatin that result in relatively long-lasting changes in gene expression without any change in the sequence of nucleotides. Within the

nucleus of every cell, DNA wraps around a complex of histone proteins to form nucleosomes. These nucleosomes are further packaged and condensed to different degrees. In general, loose packaging makes DNA more accessible to the transcriptional machinery and is associated with increased gene expression. More compact, heterochromatic states are associated with reduced gene expression. Epigenetic marks such as DNA methylation and the modification of histone tails determine the degree of packing, and therefore gene expression.[259]

Several observations suggest an important role for epigenetics in sexual differentiation. The first is simply circumstantial evidence based on how steroid hormone receptors work. Steroid receptors, including ARs and ERs, are known to recruit co-activators that increase gene expression and/or co-repressors that decrease expression. These co-factors themselves often have histone-modifying activity.[260–262] An important mechanism by which gonadal steroid hormones activate gene expression may therefore involve the opening up of chromatin structure following the binding of a receptor–co-activator complex. Based on this observation alone, one might wonder whether all effects of steroid hormones are "epigenetic" (i.e., require chromatin modifications). More directly related to the topic of this chapter, blocking these steroid receptor co-factors can prevent the effects of testosterone on morphological and behavioral sexual differentiation.[263,264] Although research into the role of epigenetics in sexual differentiation is still in its infancy, this has become an active line of research in the last few years. To date, a role for both histone acetylation and DNA methylation has been found.

Histone Modifications

The histone proteins have N-terminal "tails" that protrude from the nucleosome and can be posttranslationally modified in a variety of ways. The acetylation of histone tails is the best understood of the histone modifications. Histone acetyltransferases add acetyl groups to lysine residues of histone tails, which opens the chromatin structure, increases access for transcription factors, and enhances transcriptional activity. Histone deacetylases (HDACs), on the other hand, remove acetyl groups from histone tails, and this is generally associated with reduced gene transcription.[265,266] A sex difference in histone acetylation in the cortex and hippocampus of perinatal mice has been described.[267] This sex difference could be reversed by treating females with testosterone, so it correlates with sexual differentiation of the brain.

To more directly test whether modifications in histone acetylation were important in sexual differentiation, Murray et al. treated mice with an HDAC inhibitor during the critical neonatal period.[268] As noted in Table 47.1, the BNSTp is normally larger in volume and cell number in male than in female mice.[105] Neonatal HDAC treatment prevented masculinization of BNSTp cell number in both males and testosterone-treated females, while having no effect on cell number in control females (Figure 47.13).[268] Treatment of newborn mice with an HDAC inhibitor also had no effect on cell number in two non-sexually dimorphic brain regions in this study, suggesting that blockade of histone deacetylation did not have a generalized effect on cell number, but specifically prevented the masculinizing actions of testosterone.

More recently, Matsuda et al. used a similar approach to ask whether the masculinization of male sexual behavior in rats requires alterations in histone

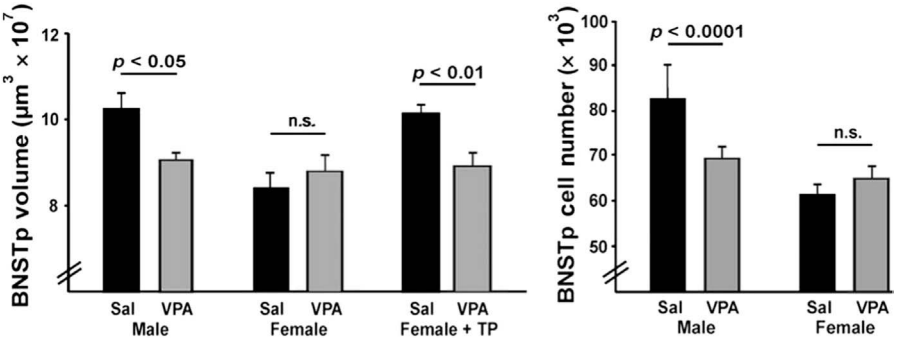

FIGURE 47.13 Treatment of neonatal mice with a histone deacetylase inhibitor prevents masculinization of the principal nucleus of the bed nucleus of the stria terminalis (BNSTp). Valproic acid (VPA), a histone deacetylase inhibitor, or saline (Sal) was given to male, female, or androgenized female mice (female + testosterone propionate) on postnatal days 1 and 2. All animals were sacrificed on postnatal day 21, and volume and cell number of the BNSTp were examined. As expected, saline-treated males and androgenized females had larger volume (left) and cell number (right) of the BNSTp than did control females (compare black bars). Treatment with VPA reduced the volume and cell number of the BNSTp in both males and testosterone-propionate-treated females but did not affect these measures in control females. These findings suggest that histone acetylation plays a role in sexual differentiation of the BNSTp. n.s.: not significant. *Source: Reprinted with the permission of the Endocrine Society from Ref. 268.*

acetylation.[269] When an HDAC inhibitor was infused into the cerebral ventricles of newborn male rats, these animals showed impairments in some aspects of male sexual behavior in adulthood. Similar behavioral effects were seen when antisense oligonucleotides to specific HDACs were infused neonatally. This latter observation is important because it strengthens the conclusion that the effects of the HDAC inhibitor were via its actions on histone acetylation, and not the result of some nonspecific action. The findings of Murray[268] and Matsuda et al.[269] are consistent in suggesting that masculinization of some aspects of neuroanatomy and behavior normally requires testosterone-dependent reductions in histone acetylation. Because reductions in histone acetylation are associated with reduced gene expression, this may mean that some gene or genes need to be turned off in males for masculinization, an interesting twist on the historical view that masculinization is an "active" event. Specific gene targets underlying the effects of the HDAC inhibitors in the studies by Murray[268] and Matsuda et al.[269] are not known. In the case of the BNSTp, since the sex difference normally arises via reduced apoptosis in males, cell death genes are predicted targets. Matsuda et al. observed dynamic, age-related changes and sex differences in histone acetylation in the ERα and aromatase genes in perinatal rats.[269] The challenge for the future is to test whether changes in histone modifications associated with specific genes are causally related to behavioral and neuroanatomical outcomes.

DNA Methylation

Changes in histone acetylation and DNA methylation often go hand in hand, with decreases in acetylation leading to increases in methylation, and vice versa. Methyl groups are added to cytosine residues on DNA, and this generally represses gene expression. A family of DNA methyl transferases (DNMTs) is responsible for placing methyl marks, and a recent study found that female rats express more DNMT3a mRNA and protein than males in the amygdala on postnatal day 1.[270] This was not seen in two other brain regions and was reversed by treating females with either an estrogen or androgen, suggesting that the sex difference in DNMT3a expression was due to gonadal steroids.

The methylation of gene promoter sites often recruits methyl binding proteins and co-repressors, which in turn cause the condensation of chromatin and decreased gene expression. In the hypothalamus and amygdala, neonatal female rats express more of the methyl binding protein, MeCP2, and nuclear receptor co-repressor (nCOR) than do males.[271,272] Taken together, several players in DNA methylation are upregulated in a hormone-dependent fashion in the female amygdala during the critical neonatal period.

In the preoptic area, however, the opposite pattern was seen: DNA methylation in the promoter region of the ERα gene was greater in neonatal males than in females.[273] This correlates with lower levels of ERα expression in the preoptic area of developing males.[143,274] Interestingly, simulated maternal grooming of females (stroking the rat pups with a paintbrush) increased ERα promoter methylation and decreased ERα expression to levels equivalent to those in control males.[273] Male rats normally receive more anogenital licking from the mother,[275] and this increases masculinization of several anatomical or behavioral traits. Thus, the findings of Kurian et al.[273] suggest molecular mechanisms whereby variations in maternal care can affect sexual differentiation of a gene that plays a crucial role in sexual differentiation of the brain.

Schwarz et al.[276] examined the DNA methylation status of promoter regions of the ERα, ERβ, and progesterone receptor genes in male and female rats throughout development. They found several sex differences that were age- and brain region-specific, suggesting dynamic regulation of methyl marks. Indeed, in at least two cases described so far (vasopressin and ERα), the gene may shuttle between methylation states, depending on testosterone status in adulthood (Figure 47.14).[276,277] Several labs are now taking a broader look at the role of DNA methylation in sexual differentiation. For example, Ghahremani et al. recently reported preliminary findings on a whole-genome survey to identify genes that are differentially methylated in sexually dimorphic regions of the mouse brain. Interestingly, the majority of the differentially methylated genes have increased methylation in males (or testosterone-treated females) compared with

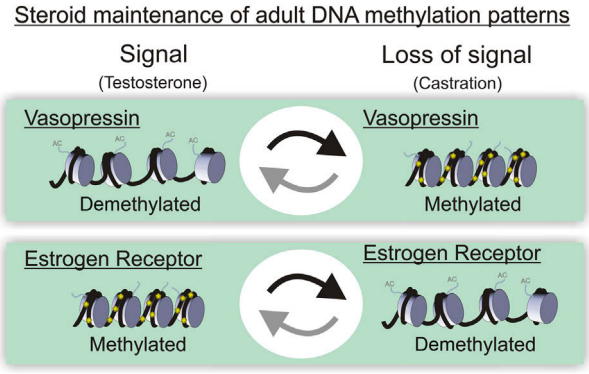

FIGURE 47.14 DNA methylation patterns within the adult brain may be more plastic than originally thought. While some DNA methylation patterns are stable, others have been shown to cycle between methylated and demethylated states. Within the adult male rat brain, testosterone regulates DNA methylation patterns of both the vasopressin and ER gene promoter regions. Loss of testosterone results in increased methylation of the vasopressin promoter and demethylation of the ER promoter. *Source: Adapted with permission from Elsevier from Auger and Auger (2013).*

females,[278] consistent with masculinization being associated with a turning off of genes, as suggested by the histone acetylation studies also mentioned here. Nugent and McCarthy[279] are taking a different approach by looking at changes in gene expression in the preoptic area of newborn male and female rats treated with a DNA methyltransferase inhibitor; any differences are presumably genes whose expression is normally regulated by DNA methylation. Although still preliminary, large-scale studies like these are likely to broaden our understanding of the contribution of DNA methylation to the differentiation of male and female brains.

See Chapter 52 for further discussion of epigenetics in reproduction.

CRITICAL PERIODS

Already in the 1959 landmark paper of Phoenix, Goy, Gerall, and Young,[28] the concept of critical periods in sexual differentiation was made explicit. Treating female guinea pigs with testosterone prenatally changed their behavioral response to hormones in adulthood, but that same effect was not seen in females treated with testosterone postnatally. These results suggested a critical window for the effects of testosterone on sexual differentiation of copulatory behavior.

Similarly, in one of the earliest reports of a sex difference in brain structure, the size of the SDN-POA, which is larger in male rats than in females (Table 47.1), was unaltered by gonadectomy or other hormone manipulations in adulthood but was reduced in males that were castrated as neonates.[67] A follow-up study was designed to determine exactly when testosterone acts to differentiate SDN-POA volume: injections of testosterone were given on single days between late gestation and early postnatal life, and adult size of the SDN-POA was measured in the resulting female offspring. A single testosterone injection on any day between embryonic day 18 and postnatal day 5, but not before or after, increased SDN-POA volume of females,[280,281] thus delineating a sensitive window for effects of exogenous steroids on SDN-POA development. One interesting wrinkle, however, is that the timing of the critical period for administering exogenous testosterone in females may not be the same as that for removing the endogenous androgens of males.[282]

The organizational–activational hypothesis of Phoenix et al.[28] went further than simply demonstrating the existence of critical periods by suggesting that effects of gonadal steroids are permanent (the organizational or programming effects of hormones) and that later in life they are transient (or activational). It is now clear that there is not a neat dichotomy between the early effects of steroids and those happening later in life.[283] Hormones acting long after the perinatal period can have lasting effects, for example, and there is no evidence for fundamentally different modes of hormone action in neonates versus adults. As far as we know, testosterone and its estrogenic metabolites bind the same receptors throughout ontogeny and trigger similar intracellular events. So, to the extent that steroids exert seemingly dichotomous action on behavior depending on whether they are present perinatally or in adulthood, it is likely due to differences in the developmental state of the tissues they act upon.

In some cases, the cellular basis for the existence of a critical period seems clear. As reviewed here, several sex differences in the nervous system depend on differential cell death in males and females, and in rodents the bulk of this cell death takes place perinatally.[177,202,284] Hormones clearly cannot bring dead cells back to life, so it is not surprising that the programming effects of steroids on cell number are time limited. Similarly, the migration of most newly born neurons to their final position in the rat brain is complete perinatally,[186,285,286] so hormones would presumably have to act pre- or early postnatally to significantly influence this process. For example, treating rats with dihydrotestosterone appears to halt the secondary migration of SNB motoneurons to a dorsomedial position in the ventral horn, but only if given prenatally, before the migration is complete.[287]

To the extent that neurogenesis and gliogenesis continue beyond perinatal development, however, the hormonal modulation of these processes could also occur later. It is now clear that in some parts of the brain, neurons are generated throughout life. To the extent that hormonal state can influence the generation or survival of these new cells, the "critical period" for hormone effects on cell number would remain open.[178,179,288,289]

Other sex differences depend on processes that are very active in development, but that nonetheless continue throughout life; the hormonal regulation of dendritic trees and dendritic spines in adulthood, discussed in this chapter, are examples. In at least one case, however, a critical period for hormone effects on dendritic growth has been described. Estradiol increases the dendritic extent of SNB motoneurons in male rats if given during the early postnatal period, but not if administered after the first few weeks of life.[290] The basis for this critical period may be the transient expression of ERs by the target muscles, which are the site of hormone action for estrogen effects on SNB dendrites.[290]

For more subtle sex differences, such as those defined not by cell number or morphology, but by gene expression, the basis of a critical period for hormone action may be less obvious. For example, early exposure to gonadal steroids can have a programming effect on the expression of steroid receptors that is not seen with later hormone manipulations.[144,291] Epigenetic marks that

determine gene expression levels are likely to play a role here. Although DNA methylation and histone modifications can be quite plastic, in some cases histone marks are very long lasting. Ghahramani et al. have recently demonstrated, for example, the presence of sex differences in DNA methylation that are due to early effects of gonadal steroids that remain evident even when circulating hormone levels are equalized in adulthood.[278]

Perhaps inspired by Phoenix et al.'s original dichotomy of "organizational" versus "activational" effects of gonadal steroids, for many years virtually all studies in the field of sex differences examined effects of gonadal steroids either during the perinatal period or in adulthood. The range of ages in between was rarely considered. More recently, however, it has become clear that manipulating hormone levels at puberty (well after the traditional "critical period" for sexual differentiation) can have lasting consequences on the brain and behavior, and consequences that are different from those seen when hormone manipulations are delayed until adulthood. In fact, as more investigators in the field have begun to recognize the importance of puberty for sexual differentiation, some have begun to refer to puberty as a "second critical period". This may be misleading, however. For example, when male hamsters are deprived of testosterone exposure during puberty, the ability to activate male sexual behavior is decreased in adulthood.[292] This indicates that pubertal exposure to gonadal steroids is required to fully organize the brain in the male direction. However, testosterone exposure is even more effective in enhancing later reproduction if it occurs somewhat earlier, that is, prior to normal puberty (Figure 47.15).[294] Thus, puberty may not be a period of renewed sensitivity, or a "second" critical period, but instead the end of a single, gradually waning sensitive period that starts perinatally.[294] In this view, puberty gains its importance not because of special hormonal responsiveness but because it coincides with a marked increase in circulating gonadal steroids over prepubertal levels.

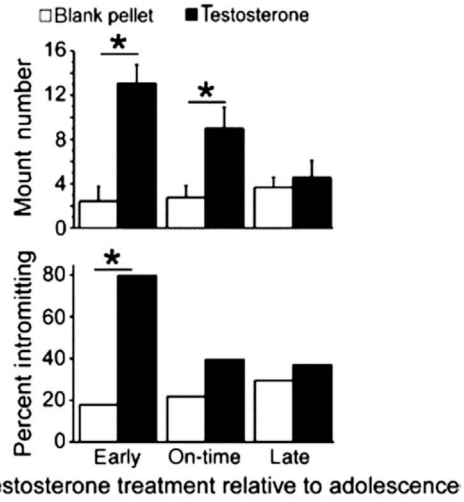

FIGURE 47.15 Effects of periadolescent testosterone exposure on adult reproductive behaviors. Testosterone treatments were designed to simulate early, on-time, and late pubertal development, and all behavior testing occurred in adulthood. Only pre- and mid-adolescent testosterone treatments increased mounts in response to testosterone in adulthood (top graph). Adult intromissive behavior was increased only by preadolescent testosterone treatments (bottom graph). These data suggest that early, preadolescent testosterone treatments may be even more effective than on-time treatments in enhancing behavioral responsiveness to testosterone in adulthood. *: Significant difference ($P<0.05$) between groups. *Source: Adapted with permission from Elsevier from Ref. 293.*

SEXUAL DIFFERENTIATION IN DIFFERENT CONTEXTS

Our understanding of the mechanisms underlying sexual differentiation of behavior rests heavily on research in laboratory rats and guinea pigs, which show clear differences in male and female sexual behavior. Mice were viewed as less useful for studying sexual differentiation in the pre-molecular biology era because behavioral sex differences in that species are less consistent. For example, some investigators find that when female mice are treated with testosterone or estradiol, they mount receptive females at similar or even higher rates than do males, suggesting that male sexual behavior is not sexually differentiated.[295,296] Other studies report that, as in rats, male mice show more masculine sexual behavior than females.[126,297]

Strain differences as well as testing conditions may explain such discrepancies. For example, following sleep deprivation, males show a stronger rebound of rapid-eye-movement sleep than females, but the size of the difference depends on whether the animals are stressed.[298] Context may also play a role in sex differences in reproductive behaviors. In one study, female rats showed a daily rhythm in estradiol's ability to stimulate lordosis behavior. As males did not show such a rhythm, the size of the sex difference in lordosis behavior changed markedly across the 24 h cycle.[299]

The conditions under which animals grow up also contribute to variability in sex differences. For example, most male prairie voles will respond paternally even when exposed to pups that they have not sired; virgin females, on the other hand, typically avoid or attack pups.[300] If females are raised to adulthood in the presence of their parents, however, they respond maternally to pups.[301] Much more subtle changes in developmental history can make a big difference: in one study, prairie voles showed sex differences in partner preference formation, or not, depending on whether they were transported by hand or in a plastic cup during routine cage changing.[302]

Studies on stress and the immune system may provide insight into the molecular processes leading to these different outcomes. In many cases, exposure to stressors during gestation and early postnatal life affects males and females differently. For example, prenatal stress typically affects males more than females.[211,303] This may create sex differences in functions and behaviors that are absent in unstressed individuals, or it may alter (usually diminish) the size of existing sex differences (Figure 47.16).[304]

One of the earliest reports of an effect of stress on sexual differentiation showed that pre- but not postnatal stress reduced male copulatory behavior and increased female-like sexual behavior in intact male rats.[305] Other groups have found similar, although not always identical, results (e.g., Refs 306,307). For example, Dahlöf et al. found that prenatal stress increased feminization, but did not affect masculinization of rat sexual behavior.[306] Physical stressors may also affect sexual differentiation: hypoxia during the last week of pregnancy reduced male rat sexual behavior, but did not affect female sexual behavior.[308]

Stress has also been mentioned as a factor in human homosexuality. Based on reports of a higher number of homosexual men born in Europe during, and shortly before and after, World War II compared to other times, and on questionnaire reports of homo- and heterosexual men about stressful events that their mothers may have been subjected to during pregnancy, some suggested that maternal stress increases the incidence of homosexuality.[309,310] Other studies, however, do not find such a link. For example, there was no significant association between homosexuality or gender identity and exposure to starvation in utero during the Dutch famine in World War II.[311] Differences in study design and quite possibly the nature of the stressors may explain differences in results. Other studies that assessed stress proneness of mothers and mothers' self-report of stress during pregnancy did not find an association between prenatal stress and homosexuality, although in one study prenatal stress predicted increased effeminate behavior among boys.[312] Another study did not find a link between prenatal stress and gender role behavior, but did find that smoking and alcohol use during pregnancy, physical stressors in their own right, were probably a factor.[313] Factors influencing human sexual orientation are discussed in greater detail in the section The Role of Hormones and Other Biological Factors in Sexual Differentiation of Human Behavior.

In addition to reproductive behaviors, exposure to stressors may also affect other sexually dimorphic behaviors. For example, prenatal stress reduces play behavior in male but not female rats.[314] Learning deficits and reductions in hippocampal neurogenesis, long-term potentiation, and dendritic spine density in the prefrontal cortex are more readily seen in prenatally stressed

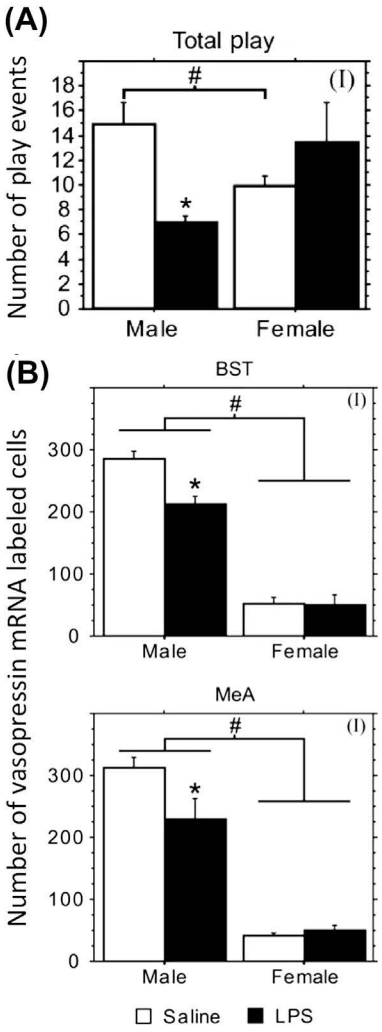

FIGURE 47.16 Effects of a prenatal immune challenge on the sexual differentiation of juvenile play behavior and vasopressin innervation of the brain. Pregnant rats were treated with the bacterial endotoxin lipopolysaccaride (LPS) on gestational day 15, and offspring were tested for juvenile play behavior between postnatal days 26 and 40. (A) The mean ± SEM number of total play events displayed in a 10-min testing period with sexually dimorphic control juveniles, with males playing more than females (white bars). Treatment with LPS reduced play in males, thereby eliminating the sex difference in play in those animals exposed to a prenatal immune challenge (black bars). (B) Following play testing, brains were collected, and the number of cells expressing vasopressin in the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA) was determined by in situ hybridization. There was a sex difference in vasopressin cell number in control animals (white bars). Prenatal LPS challenge decreased vasopressin cell number only in males (black bars). *Source: Reprinted with permission of BioMed Central from Ref. 304.*

males, while anxiety, depression, and increased response of the hypothalamic–pituitary–adrenal axis to stress are more prevalent in females. In addition, after exposure to a social stressor during juvenile life (repeated exposure to an older, aggressive animal), anxiety and depression were increased much more in female rats than in males, suggesting that the sensitivity of the developing brain to

stress hormones may differ in the two sexes.[315] For more extensive reviews on the effects of pre- and postnatal stress on sexual differentiation, see Refs 209,303,316–318.

Context also plays a role in sex differences in human behavior. One of the most consistent cognitive sex differences is found in the Mental Rotations Test. In this famous test, subjects mentally rotate a block figure to match it with a congruent object in a line-up of similarly shaped but not identical objects. Males outperform females on this task in a wide range of studies, and a metaanalysis concludes that females exposed to elevated androgens prenatally perform better, on average, than control females on this task.[319] But, interestingly, a much smaller male advantage is seen if, instead of interconnected cubes, the figures take on a human shape (Figure 47.17).[320] Similarly, the male advantage in certain math tests is eliminated or reduced if female subjects are told in advance that females do as well as males or better on these tests, but sex differences are exacerbated if females are told the reverse (reviewed by Spelke[322]). In all of these cases, context, whether defined as the conditions during the test or those leading up to the test, clearly determines the performance outcome. To what extent these different behavioral outcomes are reflected in context-dependent structure or function of the brain is not known.

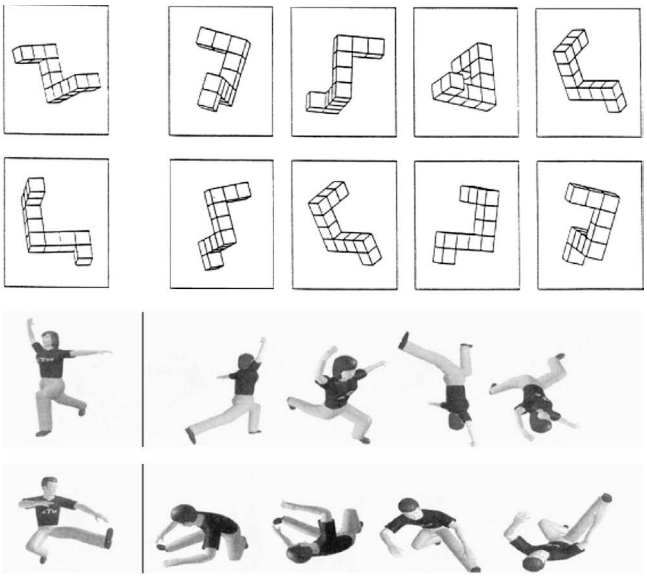

FIGURE 47.17 Size of the sex difference in the mental rotations task depends on the figures used. Top panels: samples of block figures used in the standard mental rotations task. Men generally perform better than women when these stimuli are used in the test. Bottom panels: examples of the human figures used in the modified task by Alexander and Evardone. The male advantage was reduced in this context, particularly if the figures depicted were female. *Source: Adapted with permission from Elsevier from Refs 320,321.*

THE RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION

Although the authors of the classic Phoenix et al. 1959 paper suspected that during development, testosterone permanently changed the neural circuits that drive sexual behavior, they expected these changes to be "subtle" and manifested "in function rather than in visible structure."[28] Subtle changes were indeed found soon thereafter. In 1966, Pfaff showed that neonatal castration permanently changed the size of nucleoli in hypothalamic cells.[323] Four years later, McEwen et al. showed that neonatal steroid treatment altered testosterone and estradiol uptake in rat brains, which probably reflected changes in steroid receptor binding.[324,325] The following year, Raisman and Field used electron microscopy to reveal that males have more synapses of nonstrial origin on dendritic shafts and fewer on dendritic spines in the preoptic area than do females.[326] Although we still don't know the functional implications of these synaptic differences, this was the first demonstration of a morphological sex difference. Moreover, like sexual behavior, it could be reversed by neonatal manipulations of gonadal hormone levels.[237]

It soon became apparent, however, that sex differences were present in gross morphology of the brain as well. The first such difference was found in the size of song control nuclei in the brain of canaries and zebra finches, which are larger in males than in females.[327] A big surprise was Gorski and his colleagues' discovery of the SDN-POA (Table 47.1) 2 years later,[67] considering that its dimorphism is obvious to the naked eye in an area that had been looked at for decades. These early findings opened the floodgates. A PubMed search performed during the writing of this chapter yielded nearly 4000 hits for the entry "sexual differentiation OR sex differences" *and* "brain". Sex differences have been found almost everywhere in the brain, and many of these have been detailed in more than 100 scholarly reviews, some of which we cited in the beginning of this chapter. Finding sex differences clearly is not a problem.

A much more difficult problem has been identifying the *functional significance* of sex differences in brain structure. For example, the medial POA (mPOA) has been linked to male copulatory behavior, so when sex differences were found in this region they were reasonably supposed to be related to sex differences in this behavior. Similarly, sex differences in the ventromedial nucleus of the hypothalamus were presumed to underlie sex differences in female sexual behavior, and sex differences in the AVPV to underlie sex differences in the regulation of gonadotropic hormones, as these areas are most prominently associated with these respective functions.[215,328,329] Sex differences that were found in brain areas that have not been traditionally related to reproductive functions and behaviors have in some cases also been linked to sex differences in functions associated

with these structures. Sex differences in the cortex or corpus callosum, for example, have been attributed to sex differences in cognitive abilities, once again relying upon the notion that sex differences in structure beget sex differences in function.

However, sexually dimorphic areas have also been implicated in functions that show no clear sex differences, or that show differences that are not consistent with the differences in structure, suggesting that the relationship between sex differences in structure and function is not so simple. The mPOA and its role in male coital behavior provide a good illustration. Because the mPOA is required for male copulatory behavior in most vertebrates, when the SDN-POA was discovered, it was first speculated that the sex difference in neuroanatomy might explain the difference in coital behavior. However, lesions limited to the SDN-POA, a small subset of the whole mPOA, had little if any effect on male copulatory behavior in adult rats,[330–332] suggesting that the SDN-POA is dispensable for this behavior.

There are also discrepancies between the effects of perinatal hormone manipulations on size of the SDN-POA and the masculinization of sexual behavior in rats. For example, treating perinatal males with an aromatase inhibitor decreased the size of the SDN-POA in adulthood, but had little effect on masculine copulatory behavior.[333,334] Treating females with testosterone during late prenatal life increased the size of the SDN-POA without a concomitant increase in the likelihood that they would show male sexual behavior when given appropriate activating hormones in adulthood.[335] Finally, treatment of male or androgenized female pups with antisense oligonucleotides to steroid receptor coactivator 1 (to interfere with hormone action) decreased the size of the SDN-POA with no effect on male sexual behavior.[264]

In ferrets, similar inconsistencies are found in sex differences in male copulatory behavior and POA anatomy. Male ferrets have a nucleus in the dorsal POA (the "male nucleus", or MN), which females lack, and this difference is programmed prenatally by testosterone.[336] However, lesions restricted to the MN have only a minor effect on male coital performance.[337] Moreover, treatment of female ferrets with testosterone postnatally (after the MN normally forms) masculinizes sexual behavior without inducing an MN,[336] demonstrating that the MN is not required for masculine copulatory behavior.

Although size of the MN in ferrets, and homologous sexually dimorphic structures in other mammals, can clearly be dissociated from male copulatory performance, these structures nonetheless may play a role in a sexual behavior that is distinct from coital behavior, namely, partner preference. Unlike controls, male ferrets with lesions of the MN prefer male over female conspecifics[338,339] and male over female body odors.[340] Similarly, SDN-POA lesions disrupt sexual partner preference in rats,[341] and natural variation in male versus female preference in sheep correlates with the size of a sexually dimorphic nucleus in the mPOA, the "ovine SDN" (oSDN). About 8% of rams prefer mounting male rather than female sheep, and in these male-oriented rams, the oSDN is half the size of the oSDN in female-oriented rams.[75,342] This difference cannot be ascribed to adult testosterone levels,[343] but may be related to differences in prenatal levels as prenatal exposure to testosterone masculinizes the oSDN in females.[344] In humans, the third interstitial nucleus of the anterior hypothalamus (INAH3), which lies in an area homologous to the mPOA in rats, is larger in heterosexual males than in heterosexual females.[76] One study reported that the volume of INAH3 of homosexual males was smaller than that of heterosexual males.[345] Another found a trend in the same direction.[346] Thus, at present, our best guess about the functional significance of the sex difference in size of the SDN-POA and homologous nuclei is that they relate to sexual partner preference, although admittedly most of the evidence is correlational, and we still do not know how having extra cells in that area affects the attraction to one sex over the other.

Part of the difficulty of assigning a specific function to any given neural sex difference is that most brain regions are involved in numerous functions. In addition to its role in male sexual behavior, for example, the mPOA is critically involved in osmoregulation, thermoregulation, sleep, and parental behavior, to name a few. Some but not all of these other functions also show obvious sex differences; had the SDN-POA been discovered by investigators in the sleep or osmoregulation fields, the hypotheses initially proposed and behaviors examined after SDN-POA lesions may have been very different.

Another difficulty is that many sex differences in the brain may not cause sex differences at all. In fact, they may do just the opposite; they may prevent undesirable sex differences in gene expression, morphology, or function.[347] To take a familiar example, although X chromosome inactivation is exceedingly sexually dimorphic, as it takes places in every female cell and only in female cells, it serves to make gene expression of males and females more alike. That is, a very marked sex difference is there to play a compensatory role. Similarly, some sex differences in the brain may allow the brains of males and females to function similarly despite the very different hormonal conditions they experience in adulthood. At the neural network level, most things that the brain must do (e.g., thermoregulation) must be done by both sexes. A sex difference in one node of the network (i.e., a node that is larger or more active in one sex) may have to be offset by sex differences in other nodes. When viewing any sex difference in the brain, two obligatory possibilities should therefore be considered: the sex difference may be there to prevent or to cause a sex difference in behavior or another overt function of the brain.[32,348] (Figure 47.18).

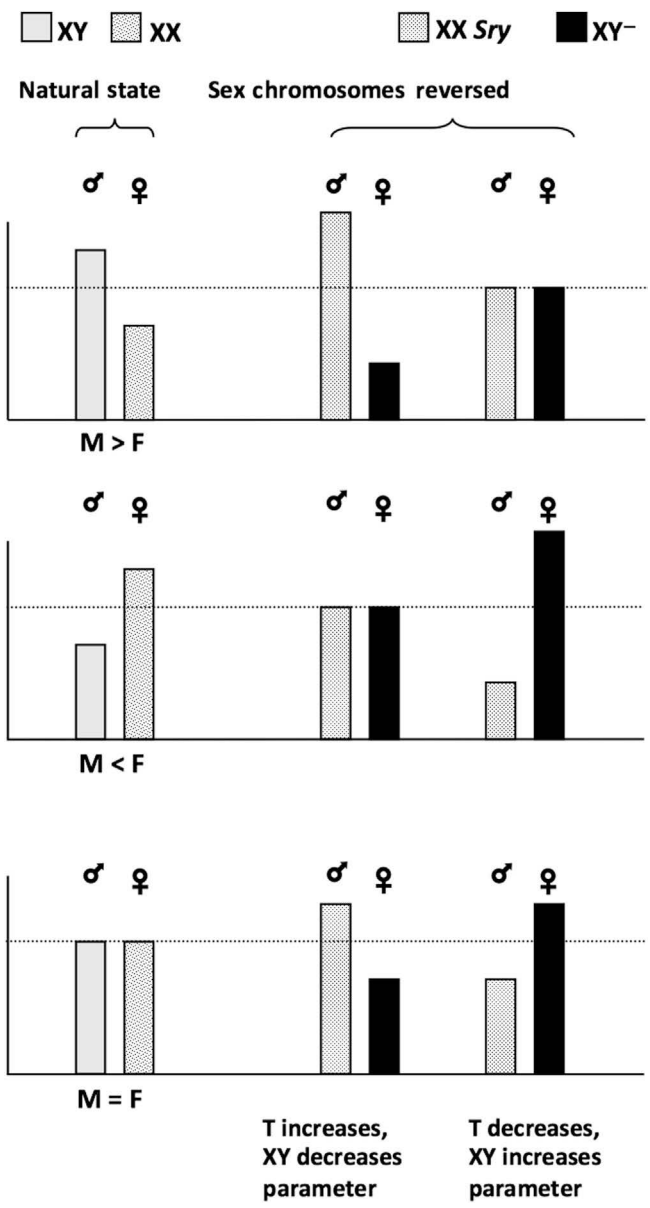

FIGURE 47.18 Gonadal steroid hormones and sex chromosomes may antagonize each others' effects or may synergize with each other in determining sex differences. The figure shows the results of a hypothetical comparison of a trait that differs between males and females, or not, in animals with normal sex chromosomes (XX in female and XY in males) or with a reversed sex chromosome complement (as in XX *Sry* males or XY⁻ female mice of the four core genotypes). The *bars on the left* (natural state) show three possible scenarios in which the parameter is larger in males than in females (M > F), is larger in females than in males (M < F), or does not differ in males and females with normal sex chromosomes (M = F). The *bars to the right* show how these parameters could differ in mice with reversed sex chromosomal complement if testosterone and the XY genotype antagonize each other's action. Under those circumstances, such antagonism may either enhance or eliminate normally occurring sex differences depending on the direction of the antagonism (either testosterone or the XY complement masculinizes the parameter). Likewise, such antagonism may induce differences in traits where they did not exist before. The male and female signs indicate gonadal sex. *Source: Reprinted with the permission of the Endocrine Society from Ref. 349.*

THE ROLE OF HORMONES AND OTHER BIOLOGICAL FACTORS IN SEXUAL DIFFERENTIATION OF HUMAN BEHAVIOR

Studies make it clear that most sex differences in the brain and behavior of animals can be attributed to one of a limited number of factors that may be considered "biological": direct effects of sex chromosomes, or the influence of hormones, principally androgens such as testosterone, or their metabolites. The extent to which these factors account for sex differences in behavior in nonhumans is really quite striking. For the most part, investigators are able to make the brains and behaviors of nonhuman subjects as masculine or feminine as they like, simply by controlling whether and when the individuals are exposed to steroid hormones. So to what extent do these same factors account for sex differences in human behavior?

Certainly there are sex differences in human behavior, but we humans have a long, protracted period of maturation, during which we incorporate tremendous amounts of information, virtually all in a social context, as we begin life utterly dependent on the nurturing of others, who are themselves acting within the confines of a rich cultural milieu. Because every human culture encountered so far promotes subtle, and sometimes not so subtle, sex differences in behavior, we cannot tell whether men and women behave differently because of the sorts of fetal hormonal mechanisms that are at work in animals, or because as boys and girls they were carefully taught, explicitly and implicitly, to behave differently. In other words, most human sex differences in behavior are equally compatible with either alternative.

As we consider various sex differences in human behavior, the question of whether, or to what extent, the differences are due to hormonal or other factors, and the alternative hypothesis of socially inculcated sex differences, will always be with us. Instead, we must rely upon correlational studies that can suggest, rather than demonstrate, whether biological factors have been at work. In addition, even when we find evidence that hormones exert an influence on behavior such that two groups of people differ, on average, in either the behavior they display or the evidence of past hormone exposure, we cannot identify any particular individual in which we can confidently say, "X hormone caused this person to show greater Y behavior".

Cognitive Function

We discussed in this chapter the sex difference in the Mental Rotations Test, which has been suggested to reflect an evolutionary history of men foraging widely for game while women stayed close to home to nurture

children. If this were indeed an evolved adaptation, one might expect the sex difference to be caused by males receiving greater fetal exposure to androgens, which notion is supported by reports that women with congenital adrenal hyperplasia (CAH), who are exposed to higher levels of fetal androgen than control women, also outperform those women on the Mental Rotations Test.[319] However, we also noted that a much smaller male advantage is seen if the figures represent humans rather than three-dimensional abstract shapes.[320]

Closely related to the sex difference in the Mental Rotations Test, at least among some theorists, is the sex difference in math performance. For many years, the average score on the mathematics portion of achievement tests such as the SAT was higher in boys than in girls. Some researchers suggested that this sex difference is independent of cultural or environmental influences. For example, in 1983, a publication in a high-profile journal, *Science*, purported to show that environmental influences were not responsible for the sex difference in SAT scores because it was also present among junior high school students who had not yet had a chance to choose elective courses in math.[350] Despite the naïve assumption that the only influence society might have on sex differences in cognitive function was through public school elective courses (ignoring, e.g., the "Baby X" experiments discussed here, suggesting social influences well before junior high school), this finding was widely reported in the national media as "proof" that sex differences in math ability are "biological", not "social".

The intervening years have not been kind to this hypothesis. First to chip away at this idea, as we mentioned here, was the discovery of stereotype threat, whereby members of a group widely considered to be inferior in some ability will display a decrement in performance on a test if they are reminded of this stereotype. In several studies, the supposed sex difference in math ability, especially at solving more difficult problems, disappears entirely if the subjects are told beforehand that there is no sex difference in performance. The sex difference disappears, not because the males' performance is affected, but because the girls' performance improves.[322] A second problem with the hypothesis that sex differences in math ability are "biological" is the fact that the sex difference in SAT math scores has been steadily dwindling over the past generation, so that it is in peril of disappearing entirely.[351] As we are aware of no change in "biological" influences on sexual differentiation of humans in that time period, the dwindling sex difference in math ability may be due to improved opportunities for women in academia and the workplace over that period. Supporting this notion is the finding that, among Western nations, those with the most gender-neutral cultures display the smallest sex differences in math performance. Indeed, in Iceland, which has a very gender-neutral society, girls now perform better than boys on tests of mathematical reasoning, on average.[352,353] Finally, if, instead of mathematical reasoning ability, we consider computational skill (basically, getting the correct answer when performing arithmetic), there has long been a female advantage.[351] To our knowledge, no one has asked whether male inferiority at this task is due to a detrimental influence of prenatal androgen. Thus, at this time it is difficult to support the idea that androgens, acting in either development or adulthood, have any effect on mathematical reasoning in humans.

There is another sex difference in human cognitive function that is much less discussed, which is the female advantage in most verbal tests. There has been less study of the causes for this sex difference, perhaps because it was smaller than the sex difference in math ability (although that may no longer be true). Because chromosomal males with androgen-insensitivity syndrome (AIS) display a female-typical ability in verbal tests, this sex difference might result from androgenic interference with verbal ability, but it could as readily be due to social influences, since XY individuals with complete AIS are typically raised as girls (see further discussion of this syndrome in this chapter). At the least, the verbal fluency of XY individuals with AIS, who also score in the female-typical range on spatial reasoning,[354] indicates that there is no effect of the Y chromosome on these cognitive differences. Interestingly, the same Icelandic study reporting that the sex difference in math ability disappeared when education became more gender-neutral also noticed that the sex difference in verbal ability increased.[352] This suggests that differential education of boys and girls may actually have served to reduce the size of this sex difference.

In short, most sex differences in higher order cognitive functions are small and getting smaller. This fact alone suggests that if these differences are affected at all by sex differences in androgen exposure, it is a small effect that would be difficult to study. However, we have yet to consider those domains that display much, much larger sex differences in human behavior: aggression, sexual attitudes, and sexual orientation. Let's consider these in turn.

Aggression

Examination of arrest records makes it clear that men are more aggressive than women, as men are over seven times more likely than women to be arrested for murder or manslaughter, and over four times more likely to be arrested for violent crime (Table 47.2). Are men more aggressive because of biological predispositions or because of cultural expectations?

TABLE 47.2 Sex Ratios in Criminal Offenses in the United States of America

Offense Charged	Male 2011	Female 2011	Sex Ratio
Total	5,910,637	2,083,579	2.8
Murder and nonnegligent manslaughter	5951	801	7.4
Forcible rape	11,934	135	88.4
Robbery	59,410	8381	7.1
Aggravated assault	200,755	58,010	3.5
Burglary	162,496	31,497	5.2
Larceny-theft	472,669	367,518	1.3
Motor vehicle theft	33,426	7450	4.5
Arson	6125	1260	4.9
Violent crime	278,050	67,327	4.1
Property crime	674,716	407,725	1.7
Other assaults	584,784	223,455	2.6
Forgery counterfeiting	28,542	17,001	1.7
Fraud	66,262	45,797	1.4
Embezzlement	5510	5565	1.0
Stolen property; buying, receiving, possessing	49,509	12,371	4.0
Vandalism	128,600	30,079	4.3
Weapons; carrying, possessing, etc.	87,682	7741	11.3
Prostitution and commercialized vice	10,439	24,497	0.4
Sex offenses (except forcible rape and prostitution)	42,872	3326	12.9
Drug abuse violations	761,050	193,114	3.9
Gambling	2238	453	4.9
Offenses against the family and children	49,408	18,183	2.7
Driving under the influence	579,176	186,459	3.1
Liquor laws	230,304	97,178	2.4
Drunkenness	303,138	66,955	4.5
Disorderly conduct	263,529	103,646	2.5
Vagrancy	14,406	3294	4.4
All other offenses (except traffic)	1,714,102	554,112	3.1
Suspicion	713	219	3.3
Curfew and loitering law violations	36,320	15,301	2.4

2011 estimated population 203,216,356.
http://www.fbi.gov/about-us/cjis/ucr/crime-in-the-u.s/2011/crime-in-the-u.s.-2011/tables/table-33.

In animal models, there is a clear influence of androgens on aggression, as castration in adulthood reduces aggressive behavior in males of many species, including rats, mice, roosters, dogs, and monkeys. In each species, exogenous testosterone treatment of castrates is sufficient to restore aggression.[355] It has been more difficult to determine whether circulating levels of androgen play a role in the greater aggressive tendencies of men compared to women. James M. Dabbs surveyed large samples of men, looking for correlates of circulating testosterone and various behaviors, occupations, and marital status, among other things. He reported that teenage delinquents and men convicted of crime had higher levels of testosterone, on average, than age-matched controls.

What's more, among men in prison, those convicted of sex or violent offenses had higher levels of testosterone than those convicted of property crimes or drug abuse,[356] as might be expected if androgens in adulthood "activate" aggression. However, Dabbs suggested that testosterone was not directly related to aggression per se, but was more closely related to dominance, since men who win chess tournaments, and spectators at sporting events whose favored team wins, also have higher levels of testosterone.[357] From that perspective, aggression might be more likely to occur among men seeking dominance over other men.

There are little or no data to address whether exposure to androgens early in life programs (or "organizes") aggression in men. Since direct measures of in utero androgen exposure are rarely obtained, available evidence rests on indirect indices. One such measure is digit ratios, which refer to the length of the second finger (index finger) divided by the length of the fourth (ring) finger. This ratio is sexually dimorphic and linked to prenatal testosterone, as discussed in detail in the section Correlates of Prenatal Androgen. Digit ratios suggest that men with indications of greater prenatal androgen exposure self-report more aggressive behavior[358] or, more specifically, aggressive *dominance* behavior.[359] Closely related to adult aggressive behavior are "externalizing behavior problems" in children. Boys with such behavior problems also have average digit ratios indicating greater prenatal androgen exposure than other boys.[360] One way in which greater prenatal androgen might increase the chance of externalizing behavior of boys, which is primarily reported in the classroom, might be by increasing attention deficit-hyperactivity disorder (ADHD). Indeed, digit ratios also indicate that boys with ADHD diagnoses were exposed to greater prenatal androgen than other boys.[361] A fascinating aspect of all of these reports of relationships between digit ratios and aggression or behaviors that might reasonably be expected to favor aggression is that they are seen only in males. None of the cited reports found a relationship between digit ratios and these behaviors in females, which suggests that a certain threshold of prenatal androgen exposure might be required before any effects on aggression-related behaviors would result.

A single functional magnetic resonance imaging study of females with CAH, which results in higher levels of prenatal androgen than in control females, supports that idea. When shown faces displaying negative emotions, adolescent girls with CAH showed a greater response in the amygdala than control girls,[362] a difference that makes them more masculine in this regard. As the amygdala has been associated with the experience of fear, it is possible that prenatal androgen above a certain threshold promotes a more fearful reaction to the negative emotions of others, which might lead to a greater propensity to reactive or impulsive aggression.

Sexual Attitudes

Perhaps the largest sex difference in any psychological trait in humans, certainly larger than the differences in math or verbal abilities, and perhaps larger than the sex difference in aggression, is the sex difference in attitudes about sexual behavior. Across societies, men report a greater interest than women in uncommitted sex, sometimes referred to as sociosexuality.[363] For evolutionary biologists, this sex difference in sexual attitudes fits well with sex differences in sexual behavior in almost all mammals, which has been explained as a result of sex differences in reproductive strategies between individuals making millions of tiny, inexpensive gametes (sperm) and those making relatively few, large, expensive gametes (ova). In mammals, reproducing via ova requires even more resources in order to nurture the offspring through an extended, internal gestation followed by neonatal lactation.

Still, given the pervasive attention of human culture to sexual behavior, the sex difference in interest in casual sex could in theory be due to socially inculcated values. On the other hand, there is evidence that circulating levels of testosterone in men correlate with interest in casual sex,[364] so that the sex difference in this attitude could theoretically be a result of the sex difference in circulating androgens in adulthood. These results also suggest that androgenic augmentation of interest in casual sex, if present, may be unrelated to any programming effect of steroids. Correlating circulating testosterone with sexual behavior in men is complicated by the well-established finding that men in stable sexual relationships have, on average, lower levels of circulating androgen than single men.[357] By definition, almost all of the married men in these samples had been, earlier, part of the population of single men, suggesting that the establishment of a regular sexual outlet reduces androgen secretion.

These two seemingly disparate findings—that testosterone boosts interest in sex, while having sex reduces testosterone—can be reconciled as a case of negative feedback. If androgen both boosted libido and was boosted by sexual behavior, that would lead to a positive-feedback system where androgens would be progressively driven higher and higher. Given the costs of circulating androgen, which inhibits the immune system and increases risky aggressive behavior, thereby increasing the risk of injury, such a positive-feedback system would be maladaptive. If so, then any analysis of a relationship between androgen and interest in casual sex would need to control for the amount of sexual activity and/or the presence of a regular sexual partner. One recent study using such an analysis found that, once the

influence of sexual activity reducing testosterone levels was controlled for, circulating levels of testosterone in men could account for over 20% of the variance in residual sociosexuality.[365] Interestingly, there was no correlation between testosterone and interest in casual sex among women, suggesting that either levels of androgen in women fall below some threshold to activate interest in casual sex, or that exposure to male-typical levels of androgen in utero is required for adult testosterone to activate these attitudes. Of course, it is also possible that cultural norms encouraging interest in casual sex for men, and discouraging it for women, mask any effects of circulating testosterone in women.

Sexual Orientation

Nowhere is the difficulty of explaining human sex differences in behavior more obvious than in the question of sexual orientation. The vast majority of men are gynephilic (sexually attracted to women), and the vast majority of women are androphilic (sexually attracted to men). Like other sex differences in behavior, this sex difference in sexual attraction is equally compatible with either hypothesis: society teaches boys and girls to develop sexual yearnings for the opposite sex, or the fetal androgens that make boys in utero also program their brains to be attracted to women later in life. But, of course, not all humans are sexually attracted to the opposite sex. While estimates vary depending on the definition, about 5% of humans report having significant same-sex attraction. Unlike heterosexuals, homosexuals contradict both hypotheses about how sexual orientation develops. They are attracted to the same sex, despite the putative influences of either fetal hormones or social upbringing. Thus, homosexuality needs explaining more than heterosexuality, but not because homosexuals are "unnatural" (whatever that means), a minority, or any more appropriately considered an object of study than heterosexuals, or because any efforts are needed to prevent homosexuality. Rather, homosexuality needs explaining precisely because it disputes the only two hypotheses that we have about human sexual orientation generally. That means that homosexuality offers us an opportunity to understand sexual orientation generally, including the vast majority of cases when people are heterosexual. If we can discern whether fetal hormones or social influences play a role in instigating those few cases of homosexuality, then we will have evidence that such influences may be at work in heterosexuals, too.

In its simplest form, an organizational or programming hypothesis of homosexuality would posit that most boys grow up to be gynephilic, and most girls grow up to be androphilic, because of their differences in fetal exposure to androgens. From this perspective, perhaps some boys fail to receive enough fetal androgen exposure to develop gynephilia, and some girls are exposed to enough fetal androgen that they become gynephilic. In other words, perhaps there is some threshold amount of fetal androgen exposure that one must receive to develop gynephilia later in life, and some boys receive less than that threshold amount, while some girls receive more than that threshold amount. On the face of it, this simplistic hypothesis is refuted by anatomy. Since almost all gay men were born with a penis, and virtually all lesbians were born without one, we know that there is no simple threshold for fetal androgen to determine gynephilia. On the other hand, it is always possible that the fetal period when androgens organize the genitalia may be different than the period during which androgens might organize the brain. So although all gay men received more androgen stimulation than any lesbians during the fetal period forming the genitalia, there may be some other period of fetal development during which the sexes do overlap in androgen stimulation. It may be during this fetal period that hormones organize gynephilia in the brain. Alternatively it is possible that the brains and genitals differ, either in their sensitivity to androgen or in the mechanisms triggered by androgen stimulation, such that a step (enzyme–protein–epigenetic modification) required for full response in one tissue has no role in the other. In this way, the same hormone level could produce a maximal response in one tissue while failing to stimulate another tissue above some threshold.

Furthermore, if we accept that human sexual orientation is influenced by many things, including genes and social influences, then it is always possible that while there is no hard-and-fast threshold for androgenic induction of gynephilia at any stage of development, it is also possible that androgen levels at some point in fetal development play a contributing (not determining) role, and that in those cases where everything else is equivalent, slightly higher or lower androgen levels than typical for that person's sex may make the difference in whether heterosexuality or homosexuality unfolds later in life.

Disorders of Sexual Development

In the absence of experimental manipulation, determining whether sex differences in human behavior result from the same sorts of processes that account for sex differences in the behavior of nonhuman subjects is not easy. We can resort to relatively rare individuals in whom biological mechanisms unfold in an atypical way and ask whether that divergent biological process is correlated with any discernible divergence of behavior. These examples of disorders of sexual development (DSDs) are well studied, so we have some data about whether they are gynephilic and/or androphilic, which might relate to their atypical production and/or

response to fetal hormones. Unfortunately, in the end there is always room for argument, as we'll see.

Through most of the twentieth century, the little evidence that fetal hormones might have any influence on human sexual orientation came primarily from the study of individuals with various DSDs. For example, in AIS, XY individuals inherit a loss-of-function allele for the AR. The Y chromosome induces the indifferent gonads to develop as testes; the testes secrete androgens such as testosterone, but the genitalia cannot respond to the hormones because they lack a functional AR. Thus, the genitalia develop in a feminine fashion, and the child is born with a feminine appearance and raised as a girl. Upon reaching adulthood, the vast majority of women with AIS are sexually attracted to men.[366,367] Note that this outcome is equally compatible with a role of either fetal androgens or social influences molding sexual orientation. We don't know if women with AIS are attracted to men because their brains could not respond to prenatal androgens to induce gynephilia, or because they were raised as girls in a culture that teaches girls to marry men one day.

Nevertheless, the androphilia of women with AIS is still informative regarding mechanisms that might be at work in human sexual orientation because it contradicts one prediction that might arise from the animal literature. In several mammalian species, including laboratory rodents, there is incontrovertible evidence that testosterone reaching the brain is often converted, via a process called aromatization, into an estrogen, which then interacts with ERs, rather than the AR, to masculinize brain structures and behaviors. As women with AIS have fully functional ERs and ample circulating testosterone to be aromatized into estrogens, their androphilia argues against the idea that aromatized metabolites of testosterone, which play such an important role in masculinizing the rodent brain, have a major role in masculinizing the human brain, at least in terms of sexual orientation. The predominant androphilia of women with AIS tells us that if fetal androgens act upon the brain at all to affect sexual attraction, they probably act through ARs, not through aromatization and ERs. Consonant with this conclusion, those few men who have been identified as lacking a functional aromatase enzyme, and are therefore severely estrogen deficient, are described as masculine in outlook (gynephilic),[368,369] as was a man who, due to a mutation of the ERα, was severely estrogen resistant.[370] The androphilia of women with AIS, who carry an XY karyotype, would also seem to eliminate the possibility that sex chromosomes play any major role in human sexual orientation.

Another clinical condition suggests more directly that ARs may affect human sexual orientation. CAH describes several different conditions, which can result from alterations in several different genes, in which a fetus is unable to secrete sufficient levels of glucocorticoids from the adrenal glands. Lack of glucocorticoid feedback to the pituitary leads to higher adrenocorticotropic hormone secretion, which causes hyperplasia of the adrenal glands and higher than normal levels of adrenal androgen secretion. Usually, the genitalia of CAH individuals are partially masculinized at birth; specifically, the clitoris appears enlarged, which leads to diagnosis and supplemental glucocorticoids to promote the child's development and to avert further androgen secretion from the adrenal glands. It is clear that girls with CAH are masculinized in terms of several behaviors, especially juvenile play behaviors.[371] It is also clear that girls with CAH, upon reaching maturity, are more likely to have a homosexual orientation than controls.[372] This outcome has been presented as strong evidence that fetal androgens affect the development of sexual orientation, but the argument is far from compelling. First, the majority of women with CAH are heterosexual,[372] despite the fact that they all have seen higher levels of fetal androgen than control women, including, presumably, many lesbians. One could argue that more of these women would be lesbians but for societal pressures favoring heterosexuality. Indeed, it is interesting that the percentage of women with CAH reporting a homosexual orientation increases as the age of the women being surveyed increases,[373] suggesting that some of these late-blooming lesbians were previously conforming to social pressures.

But, as noted in this chapter, social pressures can work in several different ways. It is difficult to assess the extent to which ambiguity about the child's genital sex might affect parental attitudes. Likewise, these girls, unlike control subjects, have a history of medical interest in their genitalia, which may include cosmetic surgery to "correct" the appearance of the pudendum, especially the size of the clitoris. Does having one's genitals regularly examined by medical staff and parents affect a child's sexual development? In fact, surveys of women with CAH indicate that few of them have sex with anyone. Of those instances, even fewer are with a female partner. For example, in one study, only 16 of the 159 women with CAH had ever had sex with another woman.[373] Is it possible that these women are sufficiently self-conscious about their genitalia, either because they are atypical (for those who did not have plastic surgery as infants) or because there is a loss of sensitivity (for those who had plastic surgery), so that they are more sexually inhibited than controls? If so, then one can imagine that a young woman who was self-conscious about her genitalia might anticipate that a lesbian partner might be more accepting of her atypical genitalia than would a heterosexual male.

Unfortunately, the relatively public nature of a CAH diagnosis inevitably affects the child's social milieu,

which may in turn have a socially mediated influence on sexual orientation. This confounding variable clouds the assessment of whether prenatal androgens affected sexual orientation in these women by acting on the brain, as the organizational hypothesis would suggest, or by acting upon the genitalia to affect the social environment, as social theorists of orientation development suggest.

The famous John/Joan case, in which an otherwise healthy male child was raised as a girl after his penis was badly damaged during a botched circumcision, was first touted as proof that social environment was sufficient to establish gender identity and sexual orientation.[366] In college psychology courses around the world, or at least the one offered to one of the present authors, the John/Joan case was presented as an existing proof that socialization was sufficient to account for gender identity and sex differences in behavior. The boy had been exposed to normal male levels of androgen prenatally, but when reared and socialized as a female, grew up to be a perfectly typical teenage girl, we were told. However, the revelation that this story was untrue threw the case in a very different light. The subject at puberty rejected his female identity, which could have been due to prenatal androgen masculinizing his brain. He took on a masculine gender identity, choosing the name David; acquired a wife and stepchildren; and embraced the roles of husband and father. But here, too, there was plenty of room for social influences, as the child was raised as a boy until 7 months of age, after which he was raised as a girl and subjected to considerable scrutiny by doctors, including annual interviews about sexual attitudes that few other children endure.[374] It seems very unlikely that the parents, having lived with a son for 7 months, would be able to unambiguously treat the child as a daughter thereafter. So was David masculine because of prenatal androgens acting on his brain, or due to incomplete socialization toward a feminine identity?

The *guevedoces*—people who are raised as girls and then, at puberty, develop masculine genitalia—are sometimes offered as evidence that prenatal androgens masculinize the brain. This syndrome results from a deficiency in 5-α-reductase,[375] which normally amplifies the androgenic signal in peripheral tissues by converting testosterone to dihydrotestosterone, which has a greater affinity for AR. These individuals are born with only partially masculinized external genitalia. The onset of androgen secretions at puberty further masculinizes the genitalia so that the phallus now resembles a penis more than a clitoris, and the swelling testes (informally referred to as "eggs" in the community) are now apparent. In the Dominican Republic community where this syndrome was first recognized, these individuals take on a masculine identity and role.[375] The simplistic presentation is that these people who are raised as girls simply shrug off social influences to become boys at puberty. The fictional presentation of such a case in the United States, in Eugenides' novel *Middlesex*, adheres closely to this idea, presumably because it was informed exclusively by the medical literature, as the author claimed to have never met anyone with this condition or any other DSD.[376]

But because the Dominican Republic community has a nickname for such individuals (*guevedoces*, or "eggs at 12"), it is hard to know how early in life they are identified by their family, society, and themselves. Thus, it is possible that these "girls" are instructed early on in their development that they will one day turn into boys. If so, then the transition could, in theory, be entirely socially mediated, independent of prenatal androgen (except to the extent that the condition sets the stage for the child to sprout the requisite "eggs" at puberty).

Because gender is such an important component of our presentation to others, any DSD that is apparent to family and physicians could, in theory, affect the perceptions of other people and thereby affect the blossoming of sexual orientation and gender identity via social influences. In the end, it is not clear whether these "experiments of nature" are truly a boon to our understanding of the ontogeny of human sexual orientation, or are "sciency" Necker cubes that are perceived by those favoring a prenatal hormone influence on sexual orientation as more compelling than they actually are.

Correlates of Prenatal Androgen

Because all of these clinical cases (AIS, CAH, *guevedoces*, and John/Joan) leave ample room for social influences on sexual orientation, there was little independent evidence that prenatal androgens, independent of socially mediated effects, influence human sexual orientation until 1998. That year, Dennis McFadden and Edward Pasanen reported that lesbians significantly differ from straight women in terms of otoacoustic emissions—sounds emitted from the ear as part of the "cochlear amplifier" that aids hearing (Figure 47.19).[377] On average, females produce more of these emissions than do males, and this sex difference is present at birth. It seems likely that the sex difference is due to prenatal androgens masculinizing the auditory system of males, reducing the production of otoacoustic emissions, because women who had a male twin have slightly masculinized levels of otoacoustic emissions.[378] Thus, the reduction in emissions from lesbians, on average, compared to straight women could be due to the lesbians receiving, on average, greater exposure to prenatal androgen than the straight women. The great strength of this demonstration was that there seems to be little room for any socially mediated factors to be at play. Almost no one knew otoacoustic emissions existed, and they are cryptic, not perceived by either the individuals or those

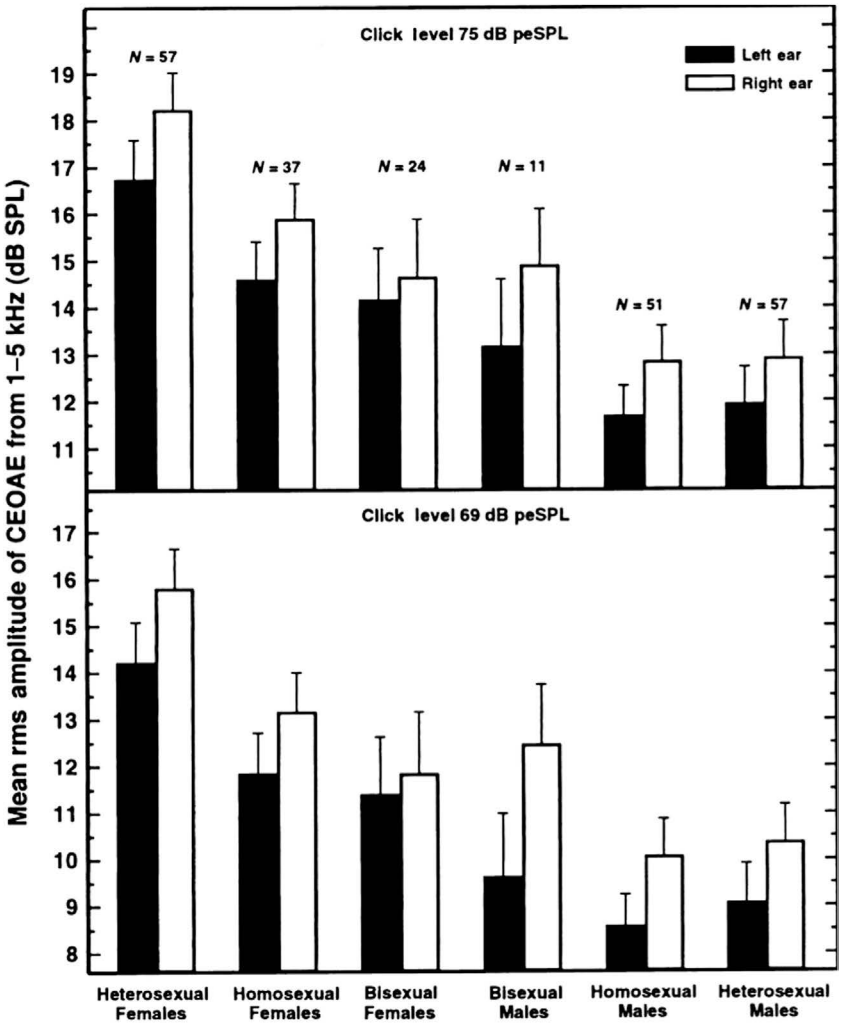

FIGURE 47.19 Otoacoustic emissions (OEAs) in response to clicks are sexually dimorphic and differ by sexual orientation in women. Shown is the root mean square (rms) amplitude of the averaged click-evoked OEA waveforms for the two highest click levels tested (75 and 69 dB peak equivalent sound pressure level (dB peSPL)), averaged across all subjects in each group. Responses to 250 clicks were collected for each click level. The error bars indicate one standard error. Amplitudes were higher for heterosexual women than for heterosexual men. OEA amplitudes of homosexual and bisexual females were reduced compared to heterosexual females, and were intermediate to those of heterosexual males and females. Sexual orientation did not affect OEAs in males. *Source: Reprinted with permission of the National Academy of Sciences from Ref. 377.*

around them. Thus, there seems little chance that any of the individuals, as children, noticed how masculine or feminine their otoacoustic emissions were, and their families surely didn't notice, either. It is difficult to conceive of any reasonable hypothesis to explain why lesbians, on average, had fewer and weaker otoacoustic emissions than straight women except that the lesbians, on average, had been exposed to greater prenatal androgen than the straight women. Nevertheless, the report from McFadden and Pasanen was subjected to a great deal of criticism, not only from the public, especially those who disapprove of homosexuality, but also among colleagues who had been studying the effects of perinatal hormones on animal brains and behavior. For whatever reasons, researchers who had placed great faith in the clinical cases, despite the inherent confounds of socialization accompanying them all, either rejected the data from otoacoustic emissions or acted as if they did not exist.

In this context, the report of a sexual dimorphism in the pattern of finger lengths that is present in 2-year-old children[379] offered a characteristic that could confirm or refute the findings of McFadden and Pasanen.[377] As described in this chapter, this sexual dimorphism in finger lengths refers to the ratio of the length of the second digit (2D) divided by the length of the fourth digit (4D). This 2D:4D ratio is greater, on average, in females than in males. It was later discovered that this sexual dimorphism is present before birth[380,381] and so cannot be caused by social influences. It is almost certainly due to sex differences in prenatal androgen exposure, because it is masculinized in people with CAH[382,383] and feminine in women with AIS.[384] The latter finding demonstrates that the sex difference in digit ratios cannot be due to sex chromosomes. In addition, in mice, in which females also display a larger 2D:4D ratio than males, AR is found in the cells of fetal digits, and several different pharmacological manipulations of AR stimulation affect digit ratios as predicted.[385] As with otoacoustic emissions, almost no one knew this sexual dimorphism existed, so it is difficult to believe that any girls' digit ratios were ever noticed by themselves or their families. Therefore, it seems unlikely that digit ratios ever influenced any social interactions.

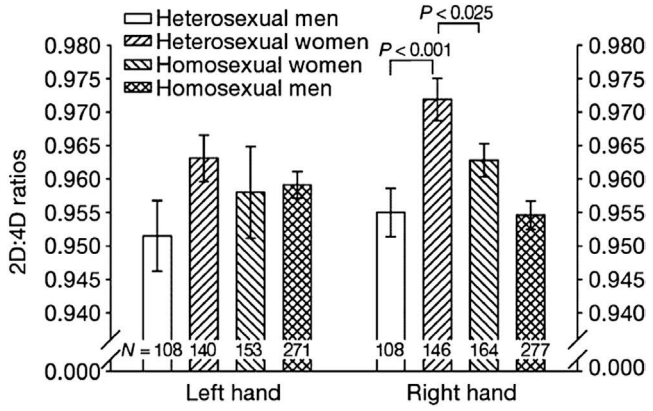

FIGURE 47.20 Finger length patterns vary with sex and sexual orientation. Among heterosexuals, the mean 2D:4D ratio is larger in women than in men, especially on the right hand. The right-hand 2D:4D ratio of homosexual women is more masculine (that is, smaller) than that of heterosexual women. *Source: Reprinted from Ref. 386, with permission from Nature Publishing Group.*

Nevertheless, lesbians, on average, have more masculine digit ratios (i.e., lower ratios) than straight women (Figure 47.20).[386] This finding has been replicated in many independent studies (but see Ref. 387) and confirmed in a metaanalysis.[388] Perhaps the most impressive replication was one with the smallest sample size—among seven pairs of female monozygotic twins discordant for orientation, the digit ratios of lesbians were smaller, on average, than those of their heterosexual sisters.[389] As with the differences in otoacoustic emissions, it is difficult to think of any reasonable explanations for why lesbians, on average, would have a lower 2D:4D ratio than straight women except that the lesbians, on average, were exposed to more prenatal androgen than the straight women. From one perspective, this finding is surprising because it is clear that at least some women are "fluid" in their sexuality, reporting having had a homosexual orientation at one period of their life and a heterosexual orientation at others.[390] Thus, it seems highly likely that some women develop a homosexual orientation that is completely unrelated to prenatal androgen. These instances of lesbianism would be expected to dilute any average difference in the digit ratios of homosexual versus heterosexual women. Indeed, in the only study that categorized lesbians, those who described themselves as "butch" had more masculine digit ratios than those who described themselves as "femme".[391]

Despite being firmly established, the difference in digit ratios between lesbians and straight women is often misunderstood, due to failure to distinguish between *average* differences between groups, and differences between particular *individuals*. The overlap in digit ratios between the sexes is extensive, as the male average is only about 0.5 standard deviations less than the female average. That extensive overlap means that one cannot reliably determine any individual's sex based on digit ratio, despite the fact that almost all females were exposed to less prenatal androgen than virtually any male. Presumably, any variation within a sex would be even less than that between the sexes. Thus, one cannot compare the digit ratios of any two individuals and reliably predict, based on that difference alone, which was exposed to greater prenatal androgen. This is even true if the two individuals are of opposite sexes! In other words, digit ratios do not offer a shibboleth to detect individual lesbians. Indeed, there is no need for such a shibboleth, as one can readily ascertain anyone's sexual orientation simply by asking them in a context where they can feel it is safe to answer.

In the meantime, several other sexually dimorphic traits, such as otoacoustic emissions discussed above, and prepulse inhibition, the phenomenon that a weak stimulus inhibits a startle response to an immediately following stronger stimulus,[392] also indicate that lesbians, on average, were exposed to greater prenatal androgen than heterosexual women. These traits also seem unlikely to directly affect anyone's social interaction and so contribute to the picture that prenatal androgen can prime the female fetus to make it more likely that she will develop a homosexual orientation by puberty. Interestingly, digit ratios, otoacoustic emissions, and prepulse inhibition, while differing between gay and straight women, do not differ between gay and straight men. This common finding across the three indices suggests that they all truly do reflect prenatal androgen or, at the least, that they all three reflect some prenatal factor.

These results in females conform reasonably well to the simple model that prenatal androgen might favor the development of gynephilia. Do digit ratios or otoacoustic emissions indicate that homosexual men were exposed to less prenatal androgen than straight men? The answer is a clear and resounding "No".[377,388] Despite the fact that gay men, on average, display less masculine play behavior as children across cultures[393] and less masculine career interests at maturity than straight men,[394] we are not aware of any morphological characteristic that indicates that homosexual men were underandrogenized in utero. In fact, if the most reliable morphological indicator of prenatal androgen influence is the genitalia, reports that gay men, on average, have a larger penis than straight men[395,396] suggest that, if anything, gay men saw more, not less, prenatal androgen than straight men.

We are left with the paradox that prenatal androgen seems to promote gynephilia in females, yet gay men, who are less masculine than straight men for certain behaviors, including gynephilia, show no somatic evidence of reduced perinatal androgen stimulation. On the other hand, there are some behaviors in which gay

men seem fully as masculine as straight men. Importantly, these behaviors are most closely associated with sexual behavior, namely, the male bias in propensity to masturbate, interest in visual pornography, interest in multiple partners and casual sex, and attraction to young, physically attractive sexual partners.[397] These behaviors are also precisely those that show the greatest sex differences among heterosexuals, far greater than the sex differences in rough-and-tumble play in children or verbal skills, math reasoning, or various memory tasks in adulthood. There is only one large sex difference in human behavior, other than gynephilia, for which gay men are reported to be undermasculinized, and that is throwing accuracy.[398] However, the sex difference in throwing accuracy could be magnified by experience if boys are more likely than girls to be drawn to activities involving throwing. Thus, the feminine throwing accuracy of gay men could well be a secondary product of their feminine play behavior and career interests, rather than a product of a deficit in prenatal androgen per se. Because gay men are hypomasculine for some behaviors but not for others, it would be difficult to see how any global deficit in prenatal androgen could be responsible.

Taken together, there is little or no evidence that gay men were underandrogenized perinatally. So it appears that the variance in sexual orientation cannot be accounted for by variance in perinatal testosterone exposure. Yet the fact is that most men are gynephilic, and that gynephilia in lesbians appears to be influenced by prenatal testosterone. How does one reconcile these findings? One possibility is that prenatal androgen does indeed foster gynephilia, and that all males receive enough prenatal testosterone to maximize whatever testosterone does to the developing brain to promote masculine sexual attitudes and gynephilia. This would explain why gay men can be so masculine in terms of sexual attitudes. What, then, can account for the variability across men in sexual orientation?

By definition, the final common pathway for testosterone's masculinizing influence, whether considering the well-established effects on genitalia or the putative effects on the brain, is the interaction of an androgenic hormone with the AR. But after that point, surely the set of AR-modulated genes that underlie growth of the penis are different from those that regulate brain development, leading, say, to a propensity to masturbate or to an interest in multiple partners or visual pornography. Presumably, there are at least some differences in the set of genes modulated by androgen even within the brain, such as in those circuits concerned with masturbation versus those concerned with young, physically attractive sexual partners. Thus, it may be that the variance in sexual orientation in men is not due to variability in prenatal androgen exposure, but to variability in the propensity of those brain circuits concerned with gynephilia to respond to that androgen exposure.

Indeed, there is ample evidence of genetic influences on human sexual orientation. Of necessity, that evidence is somewhat vague because, despite the irrefutable evidence that genes influence sexual orientation, it is already clear that this consists of a multitude of genes, each having a say, with none predominating over the others. That means, among other things, that no one has yet identified any particular gene that plays a role in sexual orientation. So it is difficult to speculate about how the genes might work. But it seems plausible that at least some of the genes, when identified, will turn out to be among those that are normally modulated by androgen influences.[399,400]

If, indeed, variability in genes that act downstream from testosterone activation of the AR accounts for variability in sexual orientation in men, then those genes will not be among those downstream from androgen's influence on masturbation or interest in casual sex. Rather, the genes modulated by androgen to affect gynephilia may also be among those that are hypomasculine in gay men—the more cognitive domains, such as verbal prowess, social acumen, spatial memory, and career interests. If correct, this reasoning may offer a strategy to identify genes involved in gynephilia by coordinating such a search with those looking for genes affecting spatial memory and social and spatial cognition.

One appealing aspect of this speculation is that it seems plausible that androgenic influences on the more "basic" aspects of male sexuality, such as a high sex drive and seeking many partners who are young and attractive, appear, in some sense, to be related to masculine genitalia. This could even be a direct effect. Perhaps it is being born with a penis, and therefore receiving the stimulation that comes from possessing an intromittent organ long before one has a chance to intromit, that leads to developing the high sex drive that males typically sport along with their penis. Ironically, this scenario resembles the sort of theorizing that was offered by Sigmund Freud, a favorite whipping boy of many psychologists. It is, in fact, a version of "anatomy is destiny". If boys, with their protruding penises, inevitably receive more genital stimulation during development than girls, and if that additional genital stimulation promotes a greater sex drive, with attendant desires for multiple partners and greater interests in visualizing potential partners, then maybe the reason why gay men share these behavioral proclivities with straight men is simply a result of something else they have in common—a penis.

In any case, if the suite of genes that promote penis development and masculine sex drive is different from those genes that promote gynephilia and the more cognitive aspects of sexual dimorphic behavior, then there is still room for more refined influences on behavior,

that is, those that result from differences solely within the brain rather than those driven by the accidents of peripheral structures. But in reality, the difference is not in terms of whether "anatomy is destiny" because anatomy matters in the brain, too. Indeed, for any behavioral differences, neuroanatomical measures, at some level of analysis (whether the number of neurons or the strength of synapses), must be responsible. The distinction then is between influences that androgen might wield over the brain indirectly, by affecting the body and therefore affecting experiences that shape the brain, and those that act directly upon the brain, altering behavioral potentials by influencing neurons directly.

Note that in this discussion we have skirted the issue of gender identity: whether one considers oneself to be male, female, or some alternative gender. For example, transsexuals are people who were born and raised as one gender but, at some point in their lives, begin to strongly feel that in fact they belong in another category. Thus, gender identity is separable from biological sex, and can also be independent of sexual orientation (e.g., not all male-to-female transsexuals are sexually attracted to men). There is woefully little research concerning any possible biological basis for transsexuality, in part because, compared to homosexuality, transsexuality is rare and has only recently come to the attention of the public, including researchers. There are reports of limbic regions that are more feminine in size and neuron number in male-to-female transsexuals than in control,[401,402] but it is difficult to eliminate the possibility that differences in exogenous steroid exposure did not affect the brain. Also, as with Swaab and Hoffman's and LeVay's findings in the early 1990s of differences in the hypothalamus of gay versus straight men,[345,403] such studies necessarily rely on postmortem materials, so that we can never follow the development of the hypothalamic region in a single individual. Thus we cannot know whether the brain difference preceded or followed the expression of transsexuality.

See Chapter 48 for additional discussion of the biological basis of sexual orientation.

WHAT WILL THE FUTURE BRING?

Although this chapter testifies to the impressive progress that has been made in the past half century in understanding sexual differentiation of the brain, many questions remain to be resolved. What follows is just a sampling.

How Pervasive are Sex Differences in the Brain?

The extent of sex differences in the brain is anybody's guess. For years, we have focused on sex differences that either are very obvious morphologically or are found in areas known to be involved in sexually dimorphic functions. From genome-wide expression studies performed in various tissues in the body, however, it is clear that we can expect sex differences in the expression of many genes, probably in just about any corner of the brain. Interestingly, these genome-wide expression studies do not suggest that the brain as a whole is more dimorphic than other tissues. In fact, in at least one study, sex differences in gene expression in the brain appeared less pronounced than they were in, for example, the liver.[174] This may be because the brain is quite heterogeneous, making it difficult for region-specific sex differences in neural gene expression to stand out when the "whole brain" is used as starting material. Gene expression studies in different brain regions and at multiple ages throughout the lifespan will help to sort out just how pervasive sex differences are.

To What Extent Does the Environment Play a Role in Sexual Differentiation?

There are more and more examples of context-dependent sexual differentiation. For example, environmental stressors, exposure to illnesses, the social environment, parenting styles, and many more factors all influence the nature and size of sex differences in behavior. Our understanding of the biological processes that mediate such environmental effects is rudimentary at best. Nevertheless, addressing this question is likely to help us identify key genes and gene pathways involved in sexual differentiation in general. For example, inflammatory processes affect sex differences, probably in part because the immune response and sexual differentiation share elements of each other's molecular machinery. Given the impressive inroads that the field of immunology has made in understanding the cellular and molecular basis of fighting foreign invaders, immunology may provide a treasure trove of ideas as to how brains sexually differentiate.

What Causes Sex Differences in the Vulnerability, Age of Onset, and Course of Disease, for Behavioral and Neurological Disorders?

Perhaps related to our ignorance of the extent of sexual dimorphisms in the brain, we have been remarkably unsuccessful in identifying the biological basis of sex differences in vulnerability to neurological and behavioral disorders, which in many cases are more spectacular than sex differences in cognitive function, nonreproductive behaviors, and the neural control of bodily functions. Understanding the extent of sex differences in the brain and the physiological significance of such functions may resolve some of these questions.

What is the Function of Sex Differences in the Brain?

As mentioned here, we do not understand the functional significance of most sex differences in the brain. Our field makes us painfully aware that finding the relation between structure and function is more difficult in the brain then it seems to be for pretty much any other tissue in the body. We pointed out in this review that sex differences may cause as well as prevent sex differences in behavior. With that perspective in mind, more progress may be made in pursuing the form–function question.

What are the Key Genes Involved in Sexual Differentiation of the Brain?

Although we have made a good start in identifying the cellular and molecular mechanisms involved in the masculinization and feminization of behavior, especially with regard to reproductive behavior in rodents, we have not made much progress in revealing the key genes or genetic mechanisms involved in sexual differentiation of other behaviors. And we have almost no insight into the molecular basis of sexual orientation and sexual attitudes in humans. In addition to identifying key genes, it is clear that epigenetic mechanisms contribute to the programming effects of steroids. It is unknown, however, whether the entire gamut of epigenetic mechanisms is used or whether gonadal hormones target a select group of epigenetic mechanisms in the process of sexual differentiation.

Putting It All Together: A Whole-Body Approach to Sexual Differentiation of the Brain

For many decades, the traditional way of looking at the development and maintenance of sex differences in brain and behavior has focused on direct interactions between gonads and the brain. Experiments outlined earlier in this chapter support the importance of these interactions. Gonadectomy and replacement of gonadal hormones during development and/or in adulthood can indeed reverse many of the known sex differences, and the presence of steroid hormone receptors in many neural areas where sex differences are found reinforces the idea that hormones directly target the brain. There are, however, many ways in which the message of the gonads can be relayed to the brain other than by a direct pathway via the bloodstream, and awareness of this is likely to become an important theme in the decades to come.

In a recent review, Arnold and Lusis introduced the term "sexome".[404] They pointed out that the function of every cell in the body can be seen as the product of an intricate network of interactions of all the different molecules that make up a cell. They define the sexome as "the sum of all sex-specific and sex-biased modulatory interactions that operate within [these] network[s]" (Figure 47.21). The sexome produces sex differences in emergent phenotypes, or it prevents such differences in other cases. These networks may be more dimorphic than one might expect. Genome-wide association studies show surprisingly pervasive sex differences in virtually every tissue studied. For example, 72% of all genes surveyed in the liver and 68% of the genes in adipose tissue show sex differences in expression level.[173] These percentages are much larger than one would intuitively expect to find based on the modest sex differences in the overt form and functions of liver and adipose tissue. Although the magnitude of sex differences in the expression of individual genes in most cases is small, the effect of all the differences combined may be quite significant. That is, there may be profound differences in the function of the molecular networks that make up the sexome, even when sex differences in individual gene expression are minimal.[404]

Differentiating factors for gene networks would be the same programming and modulating effects of gonadal steroids and sex chromosomes that we discussed in this chapter for sex differentiation of brain morphology and function. One can envision these factors to have stronger effects on some nodes in the network (e.g., genes with ER response elements in their promoter region) than in

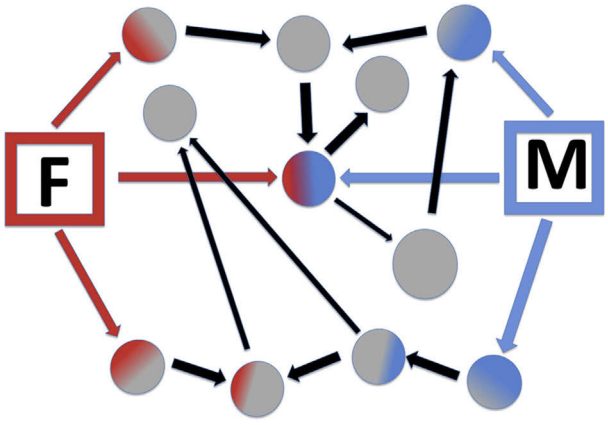

FIGURE 47.21 Schematic view of the sexome. This figure is reproduced in color in the color plate section. Male- and female-biasing factors (chromosomes, hormonal profiles, etc.) are represented by the M and F boxes, respectively. These factors act on specific nodes in the gene network causing male and female bias in gene expression represented by differently colored shading. Sex bias can be propagated to other nodes, thereby increasing or decreasing gene expression and in some cases canceling out sex-biasing effects. Arnold and Lusis define the sexome as the total of all sex-biasing actions within the network.[404] Although they focus on sex bias in gene products,[404] the circles and arrows in this figure may just as well represent interactions between different organ systems. *Source: Adapted from Ref. 404.*

others. As these genes form part of a network, however, changes in the expression of one gene will affect the expression of other genes in the network.

At its root, the sexome suggests a shift from focusing on individual genes to a more systems biology approach to sex differences. A similar type of approach can be applied to higher levels of analysis, for example by shifting the focus from individual, sexually dimorphic brain regions (nodes) to interacting neural networks. Nodes within the network participate in many, perhaps hundreds of, different functions. No single brain region can explain behavioral output, and no single region can fully explain sex differences in the functions it is involved in. But slight shifts in emphasis of one node, in combination with activity in other nodes, may have a marked effect on behavior. In other words, a full understanding of sexual differentiation of behavior may require new analytical tools, such as the use of "big data", to understand how a multitude of small changes can result in big differences in behavior or can prevent such differences.

It is unlikely that the sex differences in gene networks described here are confined to specific tissues. For example, sex differences in the function of the liver will affect the composition of the blood, and sex differences in the composition of the blood may affect the function of all other tissues in the body, including the brain. Obvious examples with respect to the topic of this chapter are sex differences in steroid metabolism and the secretion of steroid binding proteins by the liver.[405–408] Any other sex differences in the blood caused by peripheral organs may also have differentiating effects. For that matter, an initial signal from the gonads known to differentiate a neural system may have directly targeted that system but may just as well have acted indirectly by changing the production of molecules secreted by other tissues that then affect the brain. Recent work on, for example, sex differences in the cardiovascular[409,410] and immune systems[209,411] suggests that hormones acting at these peripheral sites could affect the brain and behavior. We can include the effects of prenatal androgen on the developing genitalia among these possible indirect pathways, because hormone effects on the genitalia may have a profound influence on the social influences that the individual receives, with consequences for the developing brain, as we discussed here.

It does not end there. One need not stop at other humans or human tissues as potentially mediating the sexually differentiating effects of hormones on target organs such as the brain. Recent studies suggest that the microbiome, the collection of microorganisms that live symbiotically on or within our bodies, may perform intermediary steps as well. For example, steroid hormones can influence the physiology and secretions of microbiota in our gut. Differences induced by these microbiota can influence the absorption of nutrients, secretion of lymphokines and cytokines by intestinal tissue, and even absorption of chemicals secreted by bacteria, all of which can influence the physiology of different tissues, including the brain. The interaction may operate in both directions. A recent study showed that the gut microbiome of mice becomes sexually dimorphic after puberty, likely reflecting sex differences in adult hormone levels. In turn, the microbiota influence circulating testosterone levels in male mice, and the transfer of gut microbiota from adult males to immature females alters both the hormone levels and autoimmune responses of the recipients![412]

CONCLUSION

As this review has made clear, in the 50 years or so that scientists have been actively researching the mechanisms underlying sexual differentiation of the brain and behavior, programmatic studies have revealed a more and more complicated picture. The relatively early discovery of sex chromosomes in *Drosophila* and mammals might have suggested a relatively simple process of genetic determination. In the years since, we have learned first that hormones play a crucial role in carrying out genetic instructions regarding sex, then that social interactions and other environmental factors can modulate those same hormonal messengers, with consequences for sexual differentiation. Even that view now seems somewhat dated, as we have learned that the sex chromosomes can also exert direct, non–hormonally mediated influences on the brain, and that experience can have long-lasting effects on gene expression via epigenetic mechanisms. If our vision of sexual differentiation of behavior has become more complicated, that is the price to be paid for a deeper understanding of any biological phenomenon. Rather than bemoan how many different mechanisms are at work, we can embrace the richness of the process and its deep, multilayered pervasiveness in all those vertebrate species that endure in a long succession of males and females. To borrow Darwin's phrase, there is grandeur in this view of sex.

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CHAPTER

48

Mate Selection, Sexual Orientation, and Pair Bonding

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INTRODUCTION

Sexual reproduction, as adopted by most vertebrate species, usually requires the interaction, sometimes for just a brief moment, of individuals of the opposite sexes to ensure the encounter of sperm and ova. In some cases, the interaction between the sexes will occur during a more extended period and a bond will develop between the male and the female, a bond that will last for a variable duration ranging from a single reproductive period to their entire life.

All aspects of sexual reproduction have always interested the general public, but it is only recently, around the middle of the twentieth century, that they have become the focus of scientific investigations from a physiological perspective. The selection of the sexual partner and potential formation of a bond were until recently frequently assumed to be species-specific characteristics that were essentially fixed for a given species. They were "innate" in the terminology used at that time. In 1935, Konrad Lorenz discovered, however, that some species of birds, such as ducks and geese, recognize as their own species and as potential mating partner(s) the first moving object that they see when they hatch from the egg. This aspect of mate choice is thus learned, contrary to what was commonly believed before, and other aspects of this critical behavior were later shown to be learned as well. Similarly, the sex of the subject that will be considered as a suitable sexual partner was and is often still now supposed to be fixed by some rigid, presumably "innate," mechanisms; in the human species, selection of a same-sex partner has often been considered a perversion. Under the influence of researchers such as Alfred Kinsey or William Masters and his collaborator and wife, Virginia Johnson, sex became a subject of scientific studies in the mid-twentieth century. This led to the realization that sexual partner preference or sexual orientation is actually influenced by the endocrine environment of the fetus or young infant. This aspect of mate choice is thus not as inflexible as previously thought.

The rapid technical and conceptual development of behavioral neuroendocrinology also resulted in a detailed analysis of the mechanisms that control pair bonding and to a fairly detailed understanding of why some species develop stable pair bonds whereas others do not. The pioneering work of Sue Carter and then Thomas Insel and colleagues on the endocrine control of reproduction in prairie voles (*Microtus ochrogaster*) formed the basis of the selection of this species that establishes a stable pair bond rapidly after a copulatory interaction and related vole species that do not establish such bonds as a model system to analyze the neuroendocrine mechanisms of pair bonding. This research has resulted in spectacular advances in our understanding of the neuroendocrine, neurochemical, and molecular basis of this complex behavior, with important potential implications in clinical psychology.

This chapter reviews the stunning progress that has been made in our understanding of these topics during the last few decades. The first part will be dedicated

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to mate choice; we will consider in sequence the different aspects of this choice, including the selection of a partner belonging to the proper species and to the biologically relevant sex for reproduction. A substantial section will describe the research in animals and humans that suggests, when it does not demonstrate, that prenatal endocrine factors as well as genetic mechanisms influence, but probably do not fully determine, this selection of a member of the opposite sex as a sexual partner. Most of this research in humans is obviously based on the study of subjects demonstrating the alternative phenotype, i.e., who demonstrate same-sex sexual attraction. This section will review the available and obviously incomplete evidence indicating that differences in sexual orientation can often be partially correlated with data suggesting an atypical endocrine environment during early life. The characteristics that determine or influence the choice of a specific sexual partner will be reviewed in the last part of this first section. This is by far the topic about which we know the least, but it is clearly possible to submit this question to scientific inquiries, and hopefully the summary presented here will stimulate further work in the near future.

The second part of this chapter details the research analyzing the brain mechanisms that control pair bonding, with a particular focus on studies in the socially monogamous prairie vole. This research has revealed that the neuropeptides oxytocin (OT) and vasopressin interact with the mesolimbic dopamine system to facilitate the formation of enduring social bonds between sexual partners. The role of the opiate and stress systems in pair bond formation will also be reviewed. Once a pair bond is established, it must be maintained. The roles of dynamic changes in dopamine receptors, corticotrophin releasing factor, and opiates in pair bond maintenance will be discussed. This section then will examine evidence that early life experiences can influence the ability to form social bonds in adulthood. Finally, the chapter will conclude with a comparative perspective including what is known about the physiological mechanisms related to pair bond relationships in birds, primates, and human beings.

EVOLUTIONARY SIGNIFICANCE OF MATE CHOICE

The behavior and physiology of an individual are ultimately (in terms of evolution) controlled by its inclusive fitness measured (again in evolutionary terms) by his/her capacity to pass his/her genes to the largest possible number of offspring in the next and following generations. The different factors that contribute to this fitness are often difficult to identify and even more so to quantify, but it is clear that mate selection plays a critical role. Reproduction is indeed a very costly endeavor in terms of both time and energy invested, and making the wrong partner choice will obviously have dramatic consequences on the number of surviving offspring that a given individual will produce.[1]

Many examples of costs associated with bad mate choice are immediately evident. Mating or attempting to mate with a partner of the wrong species or wrong sex will obviously not produce offspring or will produce infertile ones. More subtly, mating with a partner that is not healthy, or who will be unlikely to raise the young, will also result in decreased reproductive success. Identification of adequate conspecific mating partners is also important to maintain species isolation as well as ensure genetic diversity, which ultimately has an impact on fitness.

Note also that two broad types of reasons can lead to the selection of specific characteristics in a mate. First of all, as already mentioned, a better partner will provide better offspring (higher fitness), which will increase transmission of genetic traits of a given individual to the next generation. This is usually referred to as *indirect* benefits.[2] *Direct* benefits can also be expected from a better partner if he/she will, for example, provide more help in raising the young and thus minimize the cost of reproduction for a given individual, which could therefore increase his/her survival.[3,4] Mate choice thus has a critical role in the biology of a species, and evolution has adopted multiple mechanisms to ensure that this partner selection is made in an appropriate manner.

Selecting an appropriate partner for reproduction involves selecting a mate based on a number of specific characteristics, namely the species, the sex, and finally the specific individual. This selection involves collecting information on morphological, physiological, and behavioral features of the potential partner, analyzing this information, and finally making the appropriate decisions. These processes obviously involve most, if not all, sensory modalities (vision, audition, smell, taste, touch) and very sophisticated cognitive brain processes. The underlying physiological mechanisms have been identified in some cases, but in most cases, they remain elusive. We thus have varying degrees of information on the physiological mechanisms mediating different aspects of mate choice. We will, in this chapter, attempt to cover these different aspects of mate choice in a manner that will be as systematic and comprehensive as possible. However, in many cases, information concerning underlying physiological processes is simply not available. It will therefore be impossible to discuss these aspects. These gaps in our knowledge represent important areas

of study for future research that will be highlighted in the concluding section.

MATE CHOICE AND MATING STRATEGIES

In evolutionary terms, mate choice is tightly linked to the notion of sexual selection, a concept originally introduced by Charles Darwin. The selection of a mate will ultimately be linked to the attractiveness of its traits, and these will evolve and be selected for during the course of evolution. This often leads to the evolution of sexually dimorphic features that are highly salient to the opposite sex.

Mate choice is obviously a two-way decision process: each individual is at the same time the chooser, but is also chosen by a potential partner. When such a mate choice exists, the choice is most often asymmetrical. Charles Darwin believed that this could be expected based on the fact that the female usually invests more in reproduction and is also producing a smaller number of gametes.[5] Females would thus be the choosiest sex; males would mate with every possible female they could find and attract. These ideas would form the basis for social systems based on polygamy, or more precisely polygyny, with one male mating with many females, while each female would mate with only one male who would be carefully selected. This social system (polygyny) is broadly found in the animal kingdom but there are nonetheless many exceptions. The opposite system, polyandry, where one female mates with multiple males, has also been described, although it is by far less frequent (see Ref. 1).

A relatively small number of species, except in birds where it is common, have in contrast adopted monogamy as a mating system.[6] In this case, one male and one female pair for an extended period of time that can last from one reproductive season up to the entire lifetime. There is usually the formation of a prolonged and exclusive social bond, referred to as a pair bond. There is a wealth of knowledge on the genetic, neural, and physiological mechanisms underlying pair bond formation and maintenance, which will be described in detail in the last part of this chapter. Note also that even if a female practices social monogamy and spends most of her time associated with a single male, she can nevertheless mate with many males over the course of her lifetime. Biologists now have solid evidence that socially monogamous pairs of animals are not always, or perhaps even rarely, sexually exclusive. With the advent of DNA fingerprinting, it has become clear that even socially monogamous birds often brood and feed chicks in their nest that have been sired by multiple males; sometimes not even a single chick was born to the male who is taking paternal responsibilities.[7] This is why it is critical to distinguish sexual monogamy from social monogamy. Social monogamy is relatively rare in the animal kingdom. Only 3% of mammalian species are socially monogamous, while in contrast over 90% of birds are socially monogamous.

Monogamous mating systems are thought to evolve under two circumstances: (1) when survival of the offspring is greatly facilitated when the mother and father cooperate to raise the offspring, as in cases where males can contribute resources to the offspring or when he can defend the offspring against predation, or (2) when population density is low or other factors make it difficult to find a mate who is fertile.[8,9] Monogamy is common in birds perhaps because the male and female are equally capable of providing nutrients, warmth, and protection to the offspring, and thus both can have a direct impact on the survival of the offspring. In mammals, however, only the female can produce milk and therefore provide nutrients to the young, so the potential contribution of the male to the survival of the offspring is much more limited. Consequently, in most mammalian species, the most adaptive strategy for males is to impregnate as many females as possible. However, if there are predators in the environment that tend to raid nests, and the male can help defend the nest from predation, the most successful males (i.e., evolutionarily fit) might be those that protect the offspring while the female forages. In cases where population density is low, it would be adaptive for the male to stay in close proximity to the female partner throughout pregnancy, display mate guarding behavior, and mate with her as soon as she becomes fertile again. Males who do not choose this strategy may fail to be successful at finding a female who is unmated and fertile. As we will see in the following discussion, low population density appears to be the reason for the evolution of monogamy in prairie voles.[9]

The human species is, in this context, traditionally considered as being socially monogamous. However, this largely relates to the predominance of Western societies in the world where marriages between a single man and woman are socially enforced. When individual societies are considered, it appears that the vast majority of them (~85%) are polygamous, or more precisely polygynous.[10,11] However, it is clear that humans possess the neural capacity to form enduring pair bonds, and there is growing evidence that common mechanisms underlie pair bond formation in socially monogamous mammals and humans. Thus humans may be highly adaptable with respect to mating strategy from a neurobiological perspective. Indeed it has been postulated that pair bonding in humans evolved as a consequence of the extended developmental period of dependency of infants,

necessitating cooperation between parents to ensure survival of the offspring.

DIFFERENT FACETS OF MATE CHOICE

The choice of a mate inescapably involves selecting a specific individual, his/her sex, and his/her species. The physiological processes involved in the preference for these different traits cannot be easily separated. For the sake of simplicity, we will consider in the next three sections of this chapter these different aspects of mate choice sequentially because they rely to some extent on different mechanisms. We shall move from the more general to the more specific, and will thus consider in sequence (1) the selection of the *species*, (2) the selection of the *sex*, and (3) the selection of the *specific individual* with whom mating will take place. Many signals convey information about these three aspects of a given individual. For example, body odors (e.g., pheromones) in mammals allow the identification of the species, sex, and some individual characteristics of a given subject.[6,12] However, these different types of information are not necessarily carried by the same chemical compounds. Furthermore, the way in which these signals are decoded and the underlying neurobiological mechanisms are not necessarily the same. The amount and type of information available to an individual for these different aspects of mate choice is also extremely different. It is therefore necessary to consider them in isolation even if some interactions and cross-referencing will have to take place.

SPECIES SPECIFICITY IN MATE SELECTION

By far, most matings observed in nature occur between members of the same species. Hybrid offspring can result from two organisms of distinct but closely related parent species, but these hybrids are in most cases infertile. Mating between related species has been encouraged in captive animals because the offspring sometimes display desirable characteristics (e.g., the mule resulting from the interbreeding of a female horse and male donkey that is comparatively stronger and shows greater patience, endurance, and sure-footedness), but they are almost always infertile. Thus mating between different species either produces no offspring or infertile offspring, and this obviously has a high cost for the reproductive success of a given individual.

Nature versus Nurture

While it is generally believed that sexual behavior is instinctive and therefore somewhat mechanistic, a fair amount of learning is involved in the establishment of mature adult sexual behavior.[13] Mate choice is no exception,[14] and there are now many examples in a diversity of species indicating that animals do not spontaneously, or innately, recognize their own species and need to learn its characteristic features.

The initial recognition of this learning process crystallized with the work of the Austrian ethologist Konrad Lorenz on filial and subsequently sexual imprinting (initially described by Spalding in 1873[14]). Lorenz demonstrated that young nidifugous (precocial) birds (e.g., ducklings and goslings) will soon after hatching follow any moving object that they see in the same way as they would follow their own parents.[15,16] Objects that are capable of inducing such a response can be as diverse as a matchbox, a ball, or a walking man. This does not mean that any object is equally effective. Round objects are more likely to be followed, and the effectiveness of an object increases with its conspicuousness. There are thus clear constraints on learning (see Ref. 16).

More relevant to the focus of the present chapter, filial imprinting often leads to sexual imprinting: birds who have followed inanimate objects or members of another species during their young life will, when adult, attempt to copulate with them. Sexual attraction to humans can for example develop in nonhuman animals, including birds and mammals, as a result of having been reared from birth by humans.[15,16] This phenomenon is, for example, common in falconry birds that are reared by humans. It also takes place in many domestic animals.

Examples of effects of early experience on the type of stimuli that will later in life elicit sexual behavior are known for nearly all vertebrate groups. Male zebra finches (*Taeniopygia guttata*), a small songbird commonly used in experimental studies, mate with females with an appearance similar to the female who has reared them rather than the appearance of their parents when they are different[17,18] (see also Ref. 15).

In mammals, extensive experimental evidence has been collected, at least in some species, demonstrating a role of early learning in determining the species of the mating partner. If sheep and goats are cross-fostered at birth, but raised in mixed-species groups, their play and grooming behavior resembles that of their foster rather than genetic species. More importantly, in adulthood, cross-fostered males strongly prefer to socialize and mate with females of their foster mother species, even if raised with a conspecific (Figure 48.1). Cross-fostered females also show significant preference for socializing with females and mating with males of their foster mother's species, although this effect is weaker than in males.[19,20]

Surprisingly, even if cross-fostered animals are placed in flocks containing members of only their genetic species for 3 years, male social and mating preferences for females of their adoptive mother's species remain

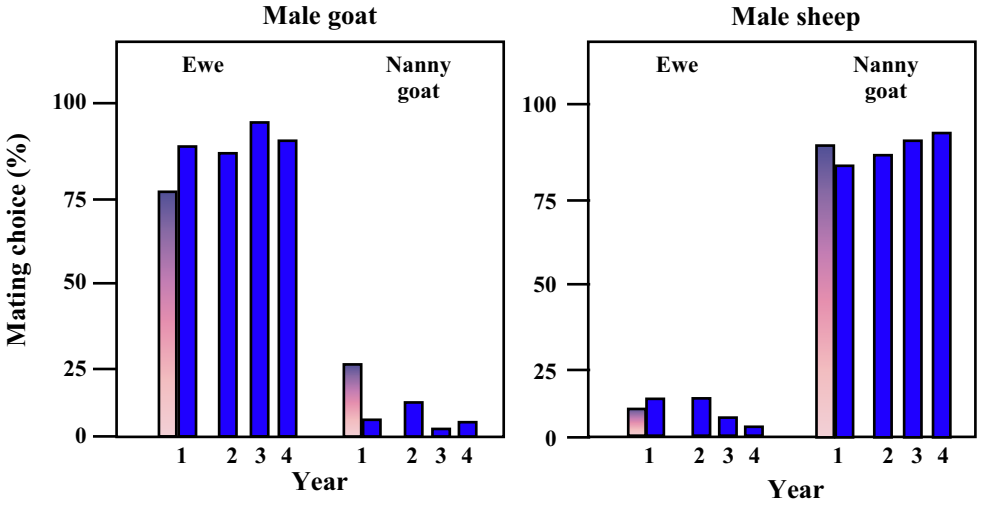

FIGURE 48.1 Percentages of mating choices made by male goats (left panel) and sheep (right panel) that had been cross-fostered at birth and raised by females of the other species. At 1 year of age, males were given a mating choice between tethered ewes and tethered nanny goats. Sheep and goats raised by females of their genetic species mated exclusively with members of their own species (data not shown). In contrast, cross-fostered males in both species mated with their maternal species. This reversed-mating choice was observed if young lambs and goats were raised alone or with a twin of their maternal species (dark column) as well as if they were raised with a twin of their genetic species (variable shaded column). Reversed choices expressed by 1-year-old males were maintained up till 4 years of age despite the fact that animals lived exclusively with members of their genetic species after year one. *Source: Redrawn from Ref. 19.*

virtually unaffected. In contrast, however, females raised in these conditions change to display an exclusive mating preference for members of their genetic species within 1 or 2 years, although they still retain some social interest in female members of their foster species.[20]

The sex difference in the magnitude of these effects of early experience suggests that they are likely affected by reproductive hormones. However, castration of male sheep and goats within 2 days of birth was shown to reduce only slightly the level of this altered social preference, and mating preference following short-term testosterone treatment in these males was the same as for gonadally intact animals.[20] The social preferences of these castrated subjects were also similar in the absence of testosterone treatment. The mating preferences in these species thus seem to be affected only marginally by pre- and/or early postnatal effects of sex steroids on the brain, but specific mechanisms, including site of action, remain unknown at present.

Early imprinting thus seems to have long-term effects not only on the object toward which the young animal will direct its following responses but also its adult sexual responses later in life. However, the juvenile experience is often in itself insufficient to determine mate preference in adulthood. The evidence supporting sexual imprinting indeed concerns only a limited number of species, and one must be careful before generalizing. Social interactions during adolescence seem to be equally important in terms of influencing mate preferences (see Ref. 16 for review). It is also clearly the case that learning does not determine all characteristics that will be recognized as species specific, and the preference for some features signaling species-specific information are surely hardwired. However, the data are currently lacking to evaluate in a general fashion the relative importance of learning versus "innate" (genetic) aspects of species recognition in the context of mating.

Sensory Modalities

The identification of the species-specific features of suitable mating partners is based on multiple sensory modalities that must in many cases interact. Additionally, it is often difficult to separate the different types of information conveyed by a given signal. A given pheromone may, for example, indicate at the same time the species, the sex, the social status (dominant-subordinate) and even the individual identity of the subject.[12] In a small number of cases, species identity has, however, been linked to a specific signal. We are providing a few select examples in the section that follows.

Vision

Because young ducks and geese may be imprinted on moving inanimate objects, it appears obvious that the stimuli that they recognize have, at least in part, a visual nature. In adult populations of ducks, males are usually more numerous than females, possibly due to the higher predation risk associated with nesting. Males engage in very extensive courtship displays to try to attract a

female and form with her a pair bond that will last for the entire reproductive season. These courtship displays were originally described by Lorenz and are highly stereotyped. The displays are shared by most species of surface-feeding (dabbling) ducks, although each individual species produces its own set of specific displays. Lorenz also discovered that different species (e.g., mallard, gadwall, green-winged teal) produce these displays in a particular sequence that is also species specific.[21–23] Furthermore these displays and their sequence are genetically inherited as demonstrated by cross-breeding experiments between several species.[22] Lorenz speculated that these sequences of fixed-action patterns (the displays) serve as a species-specific feature that prevents mating between different species. The brightly colored plumage of the males could also serve the same purpose.

Similar data are available in mammals. For example, an anecdotal study of three macaque monkeys cross-fostered at birth to mothers of another macaque species showed that as an adult, one of these monkeys preferred pictures of members of its adoptive rather than its genetic species; the study unfortunately made no direct assessments of social and sexual preferences.[24] It should be repeated in controlled conditions with a larger sample size and a formal control group.

In the cross-fostering experiment between goats and sheep described herein, normally raised male and female sheep and goats were also shown to initially approach and maintain proximity with pictures of the faces of female members of their own species, whereas in cross-fostered animals, males showed an almost exclusive preference for faces of female members of their foster species, but in females, faces of the two species were chosen equivalently.[20] It could thus be inferred that visual stimuli play a critical role in the selection of the mating partner in these species. These data, however, do not prove that these visual stimuli are the critical feature that determines the mating partner. Pheromonal signals are also likely to be involved, and more work would definitely be warranted on this topic.

Audition

Many animals are obviously capable of recognizing vocalizations of their own species. Songbirds even possess in one of the nuclei that processes their vocalizations, called nucleus HVC (a structure located in the dorsal telencephalon, originally erroneously called hyperstriatum ventrale pars caudale and subsequently high vocal center; HVC now stands as a proper name and is no longer used as an acronym[25]), sets of neurons that are specifically tuned to features of the conspecific vocalizations and even the bird's own song.[26,27] These vocalizations play a significant functional role in the biology of the species. For example, if a male great tit (*Parus major*) is captured and removed from his territory, this territory will be invaded by neighbors within minutes. It is sufficient to broadcast through loudspeakers songs of great tits to protect the territory.[28] Demonstrations that conspecific vocalizations play a key role in mate selection are rarer but nevertheless do exist.

In Japanese quail (*Coturnix japonica*), the playback by a loudspeaker of unmated male crows, a form of calling, is sufficient to attract a female. Females also showed a significant preference for a speaker broadcasting crows versus a similar speaker broadcasting reversed crows (same call but played backwards). Thus female quail, but not males, exhibit positive phonotaxis to the crows of the male.[29]

Similarly, many rodent species (including laboratory rats and mice) produce ultrasonic vocalizations during their sociosexual interactions.[30,31] It has been observed that male mice increase their ultrasonic vocalizations when exposed to females. Playback experiments with reproductively active females demonstrated that these vocalizations elicit approach behavior from the females. It is thus likely that these sounds play a role in social and species recognition, although to our knowledge this has not been fully demonstrated.

Olfaction

Species recognition may also be mediated by chemical (olfactory) signals. Animals are thought to learn at an early age the olfactory characteristics of their species in a manner analogous to visual imprinting.[32]

In 1965, Mainardi and colleagues demonstrated that female mice raised by parents scented with Parma Violet perfume preferred, as adults, a Parma Violet scented sexual partner to a nonscented one, whereas control females did not show this preference.[33] This finding was more recently confirmed by Alleva and colleagues.[34] Early olfactory learning thus has long-term consequences on mate choice.[35]

Accordingly, cross-fostered pygmy mice and house mice,[36] and cross-fostered hamsters of different species,[37] exhibit increased preferences for odors of the foster parent species later as adults. Similarly, it was shown that a male black-tailed deer raised with a surrogate mother scented with the rump gland secretion of a female pronghorn antelope will later prefer to associate with pronghorn rather than with a female of their own species.[38] A variety of studies have confirmed effects of early olfactory learning on olfactory preferences in vertebrates (for review see Refs 39,40).

SEXUAL PARTNER PREFERENCE AND SEXUAL ORIENTATION

Once a group of same-species congeners has been identified, it is still critical to select individuals of the opposite sex as mating partners in order to ensure

successful reproduction. This choice of the opposite sex for a mating partner is a sexually differentiated trait in all animal species that reproduce sexually, including humans. Most animals and people are indeed sexually attracted to individuals of the opposite sex; they are heterosexual, and this obviously conditions the success of their reproduction. In humans and in some other animal species, there is, however, a significant minority (3–10%) of subjects who are attracted to subjects of the same sex; they are homosexual. Viewed in this way, sexual orientation (hetero- versus homosexuality) is thus a behavioral feature that displays one of the largest degrees of sexual differentiation between males and females, since more than 90% of males are attracted to females (they are gynephilic or gynecophilic) and more than 90% of females are attracted to males (they are androphilic). Note, however, that an alternative view supported by a few researchers is that subjects of both sexes prefer to mate with subjects of the opposite sex, in which case orientation would be the same in both sexes (i.e., toward the other sex), and the sex difference would rather concern the sex of the subject and its perception (see Ref. 41).

The specific mechanisms that determine sexual orientation have been the subject of intense investigations and heated controversies, at least as far as humans are concerned. The focus of these disputes has usually been on homosexuality, since this orientation is less common and considered by some as "abnormal". It should instead be considered that homosexuality and heterosexuality are simply the two extremes in the natural variation of a phenotypic characteristic called sexual orientation. All intermediates do exist as already recognized in the work of Kinsey. It also must be noted that trying to understand the origins of homosexuality essentially represents the same problem as trying to understand heterosexuality: it is the question of the determinism of sexual orientation, i.e., the choice of the sex of the preferred mating partner.

Sex differences in behavior can either be learned or have a biological origin (genetic differences or endocrine controls). Although it is often assumed that human sexual orientation is determined by interactions with the parents (e.g., Oedipus complex in the theories of Freud) or by early sexual experiences (e.g., influence of the first sexual activities), little experimental evidence supports this common belief. In contrast, work in humans and various animal species has identified significant endocrine and genetic contributions to the determination of this aspect of mate choice.

Much is known on this topic concerning the sensory modalities implicated, endocrine controls, and even in some cases, brain mechanisms. We provide in this section a concise overview of the current knowledge of sexual orientation. More detailed reviews of this topic with a particular focus on humans were published recently.[42,43]

Endocrine Control of Sexual Partner Preference in Animals

Many behaviors in animals are sexually differentiated and produced preferentially or exclusively by one sex. It was originally believed that these behavioral differences between males and females were the result of the presence of different hormones in the adults of the two sexes: testosterone in males and estradiol (plus progesterone) in females.[44] Males and females indeed secrete different amounts of these steroids, and these differences in endocrine milieu could differentially activate a variety of behaviors. This is sometimes the case, and, for example, treatment of females with testosterone will activate crowing in quail, singing in canaries (*Serinus canaria*) and some degree of mounting in rats.[45]

Many behavioral sex differences are, however, not controlled by differential activating effects of steroids but rather by their perinatal action, which irreversibly differentiates the male and female brain and behavior, referred to as organizational effects.[41,45–49] Many sexually differentiated behavioral responses thus result from the early action of sex steroids that differentiate the brain along the male or female path. These differentiating effects occur very early in life, during the embryonic period or just after birth, and are, in general, completely irreversible.

In mammals, early exposure to testosterone produces a male phenotype: the behavioral characteristics typical of males are strengthened (masculinization), and the ability of males to show behavior typical of females is reduced or lost (defeminization).[41,49] The female phenotype develops in the apparent absence of hormone action (or in the presence of very low estrogen concentration; see Refs 50,51). In birds, by contrast, it appears that the male sexual phenotype (presence of mounting and copulatory behavior) develops in the relative absence of sex steroids and that embryonic ovarian steroids demasculinize this behavior in females. Female receptive behavior is not, or is only marginally, differentiated.[45]

It is therefore legitimate to ask whether the sex difference in partner preference (i.e., sexual orientation) is controlled by activational effects of sex steroids or, alternatively, is organized by their perinatal organizational action.

Activational Effects of Adult Hormones

The fact that sexual partner preference is sexually differentiated has been originally considered as evidence that it is controlled, like many other behavioral differences, by adult sex steroid hormones (e.g., testosterone and estradiol). Based on the observation that sex steroids control the activation of sexual behavior in animals and in humans, it was originally hypothesized that modifications in circulating concentrations of

these hormones were involved in the control of sexual preferences. Specifically, early investigators originally suggested that excessive concentrations of estradiol in men could be responsible for their sexual attraction to other men (a female characteristic), and that vice versa, high concentrations of testosterone induce female sexual attraction toward other women (a feature normally observed in men).

Many studies compared the concentrations of circulating sex steroids in men and women in relation to their sexual orientation, and this work clearly concluded that there are no hormonal differences between homo- or heterosexual men nor between homo- or heterosexual women.[52] The same conclusion has been reached in a few animal studies.

Human sexual orientation is thus not linked to an inadequate activation by sex steroids. This is consistent with the fact that all endocrine manipulations that attempted to modify the sexual orientation of homosexual subjects, including castration and hormone therapy, have always failed to have any significant effect. This conclusion is also in agreement with animal studies demonstrating that (1) the type of behavior patterns expressed by male or female rodents and the sex of the preferred sexual partner are not affected by steroid action in adulthood but rather depend on the early organizing effects of these steroids, and (2) circulating testosterone concentrations are not altered in male-oriented ("homosexual") sheep as compared with female-oriented ("heterosexual") subjects (see following). Adult sex steroids regulate sexual motivation but do not affect the sexual orientation of the behavior.

Organizational Effects of Embryonic or Perinatal Hormones

ORGANIZATIONAL EFFECTS OF STEROIDS ON SEXUAL PARTNER PREFERENCE IN ANIMALS

The organizing effects of embryonic sex steroids that were originally described concerned exclusively the type of behaviors (male- or female-typical) displayed by adult subjects. Studies carried out essentially during the last 20 years have, however, shown that sexual orientation (preference for a male or female mate) is also determined by prenatal hormones.

In mammals, exposure to testosterone (or its metabolite estradiol) induces male-typical orientation (preference of female over male sex partner), while in the absence of high concentrations of these steroids (or of their action), a female pattern of sexual orientation will develop (preference for male partner). A male- or female-typical sexual orientation (selection of female or male, respectively, as a sexual partner) can be experimentally induced by injections at the proper time (days or weeks preceding or immediately following birth, depending on the species) of sex steroids, or of blockers of sex steroid action. The sexual orientation determined at that time subsequently appears to be a stable characteristic of the individual.

This mechanism that now appears to apply to multiple species of rodents, and possibly more broadly, was originally identified in rat studies performed by Bakker and Slob.[53,54] It had been clearly established that during the perinatal period, testosterone masculinizes and defeminizes sexual behavior by acting essentially through its estrogenic metabolite, estradiol, produced in the brain by the enzyme aromatase.[55,56] Bakker and Slob demonstrated that the injection of an aromatase inhibitor during the end of embryonic life, or during the first week of postnatal life, blocks the development of the heterosexual partner preference in male rats.[53,54] Rats that were treated in early life with an aromatase inhibitor spent, as adults, unlike normal rats, the majority of their time in the compartment of a three-chambered arena containing another male, and largely ignored the compartment containing a sexually receptive female (Figure 48.2(A) and (B)). They also displayed lordosis behavior (i.e., sexual receptivity)

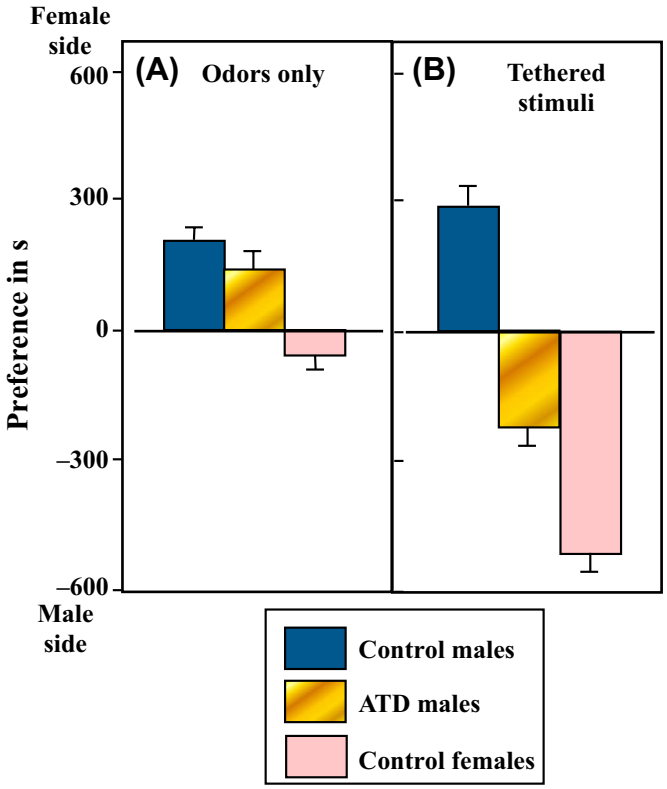

FIGURE 48.2 Perinatal inhibition of aromatase reverses the adult sex-partner preference of male rat expressed as time (seconds) spent in the female compartment minus time in the male compartment when subjects were tested with sexual odors only (A) or with tethered live subjects as stimuli (B). Experimental subjects were control males and females and males exposed to the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) pre- and postnatally.[56]

when faced with another male and allowed this male to mount them, a behavior never observed in male rats that did not receive this perinatal treatment.

The brain of these male rats displaying a sex-reversed partner preference also responded in an atypical manner to the presentation of olfactory sexual stimuli. In control animals, the nuclei that process olfactory information related to sexual behavior are activated (as measured by induction of the immediate early gene *c-fos*) in males following presentation of female-typical odors (e.g., cage bedding soiled by female urine), while the female brain is activated by the odor of males. In contrast, the brain of a male is not activated by the presentation of a litter soiled by a male and vice versa. This is very different in male rats treated during the perinatal period with an aromatase inhibitor—their brain is highly activated by the odor of other males.[57] The experimental manipulation of the early hormonal environment has thus produced a profound change in the reaction to olfactory sexual stimuli of the neural circuit controlling sexual behavior.

More recently, similar experiments confirmed that the partner preference of females is also largely determined by their early hormonal environment. Treatment during the first 3 weeks of life of young female rats with an estrogen (estradiol benzoate) increased their preference for females, a male-typical characteristic (Figure 48.3).[58] These preference tests were conducted at the adult age in ovariectomized females exposed to various hormone treatments (estrogen alone or estrogen plus progesterone) to activate different aspects of sexual behavior. The preference reversal was observed in both hormonal conditions, which is in agreement with the conclusion presented in the preceding section that sexual partner preferences cannot be modified by hormones in adulthood.

These observations initially performed in rats were confirmed in mice, but in mice androgens themselves seem to play a more important role in the differentiation of sexual partner preferences. Sexual differentiation is deeply disturbed in mice suffering from the testicular feminized mouse (tfm) mutation of the androgen receptor that prevents the action of testosterone or the nonaromatizable androgen, dihydrotestosterone (DHT).[59] In adulthood, these tfm male mice, when gonadectomized and treated with estrogens to activate sexual behavior and motivation, have responses similar to those of females. For example, they spend more time investigating, like females, bedding soiled by the urine of males, whereas control males prefer bedding soiled by the urine of females. Moreover, females and tfm males show no preference for a partner of one sex or another in simultaneous choice tests, whereas control males show a strong preference for a female partner. Finally, exposure to bedding soiled by males (but not to clean bedding) induces neuronal activation in the preoptic area and the nucleus of the stria terminalis (as measured by an increased expression of the *c-fos* gene) in females and tfm males but not in control males. The same researchers also demonstrated that treatment of neonatal females with DHT masculinizes for life all of these behavioral features and the response of the nervous system to male odors. Estrogens are, however, also implicated in the sexual differentiation of mice as they are in rats.[60,61] Together all these findings support the idea that sexual partner preferences are controlled during perinatal ontogeny by the action of sex steroids, testosterone, and its estrogenic metabolite, estradiol.

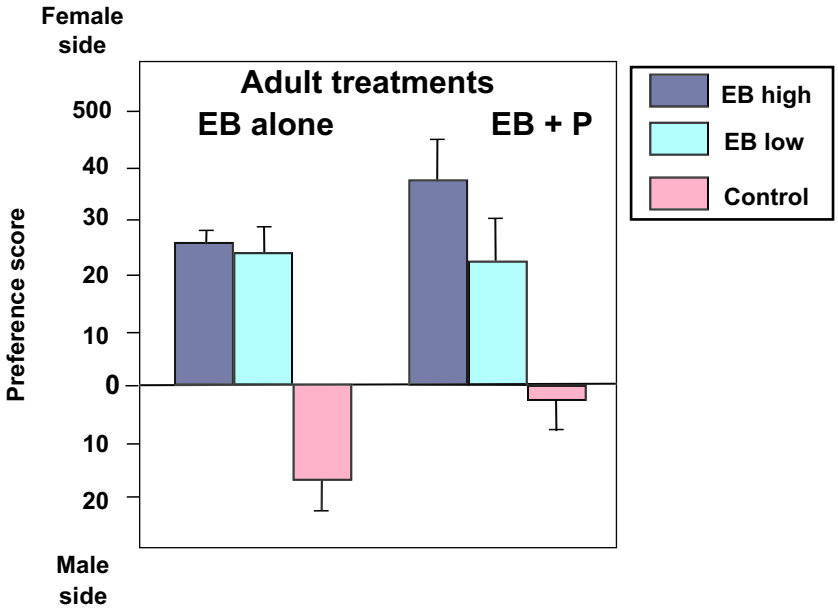

FIGURE 48.3 **Effect of treatment with estradiol benzoate (EB), at high or low dose, during the first 3 weeks of life on the sexual preferences of female rats.** The preference scores represent the time spent by the animal in the test chamber containing a female minus the time spent in the chamber containing a male during tests performed in adult ovariectomized subjects treated with estradiol benzoate (EB; left) or with EB plus progesterone (EB + P; right). A negative score indicates a preference for males (usually observed in control females). A positive score indicating a reversal of this choice (preference for females) was observed following treatment with EB at both doses used. *Source: Redrawn according to data in Ref. 58.*

Importantly, it cannot be overemphasized that these early hormonal manipulations have, as far as we know, completely irreversible effects on both the type of sexual behavior that will be displayed in adulthood (male- or female-typical) and its orientation (homo- versus heterosexual). In particular, these behavioral characteristics cannot be modified by hormonal treatments performed in adulthood.

BRAIN MECHANISMS

The medial preoptic area appears to be (one of) the likely site(s) of action of these early endocrine treatments for the control of sexual partner preference. Like rats and many species of reptiles, birds and other mammals, including humans[62,63] and ferrets (*Mustela putorius*) also have a sexually dimorphic nucleus in their medial preoptic area (SDN-POA); the volume of this nucleus is significantly larger in males than in females.[64]

Baum and his colleagues tested the effects of lesions aimed at this nucleus on male sexual partner preference, as measured in a three-compartment arena containing, in the two outer compartments, a male or a sexually receptive female as stimuli. After bilateral electrolytic lesions of this nucleus, male ferrets who previously were spending most of their time in the compartment containing a female displayed a nearly complete reversal of this choice, and spent during the postlesion tests most of their time in the chamber containing a stimulus male, a typically female preference[65] (Figure 48.4). Unilateral lesions, or lesions that missed their target, did not affect the partner preference of the males, demonstrating the anatomical specificity of the effect. Similar results have been reproduced in rats.[66] Male sexual preferences thus seem to be controlled to a large extent by the medial preoptic area. It is, then, remarkable that the organizing actions of embryonic steroids on sexual behavior and its orientation are paralleled by irreversible changes in the volume of this nucleus and other sexually differentiated structures in the brain.

The volume of the SDN-POA of rats is five to six times larger in males than in females, and this difference results almost exclusively from the action of testosterone and its estrogenic metabolites during late embryonic life and the first days of postnatal life. Once acquired, the size of the SDN-POA, which is characteristic of one sex, cannot be altered in adulthood by steroid hormones.[67,68]

It was shown that male rats treated perinatally with an aromatase inhibitor have, as adults, a small SDN-POA that is characteristic of a female.[69] As described in the previous section, these rats are behaviorally not masculinized and prefer other males over females in the three-compartment test arena. These rats also rarely attempt to mate with sexually receptive females that are present and allow stimulus males to mount them. They are showing a form of partner preference that would be qualified, in humans, as homosexuality, or at least bisexuality. Therefore, it appears that the deprivation of estrogen action during the perinatal life of male rats has a lasting effect on their sexual preferences and correlatively on the volume of their SDN-POA.

A PHYSIOLOGICAL MODEL OF ANIMAL HOMOSEXUALITY: THE DOMESTIC SHEEP

The studies discussed so far concern experimental models of homosexuality in animals based on early (perinatal) endocrine manipulations or lesions of the preoptic area. Spontaneous homosexual behavior (male mounting another male or female mounting another female) is observed quite frequently in a broad variety of animal species.[70,71] However, most often, these behaviors are only expressed when a suitable partner of the opposite sex is not available due to captivity (zoo or other captive populations), to a skewed sex ratio in the population, or to the lack of opposite sex partners because they are monopolized by dominant males (e.g., in monkey societies). These homosexual behaviors thus do not represent the expression of a preference for a partner of the same sex but simply serve as a channel for reducing sexual motivation that cannot be satisfied with the desired sex. In some cases, they also reflect a different nonsexual

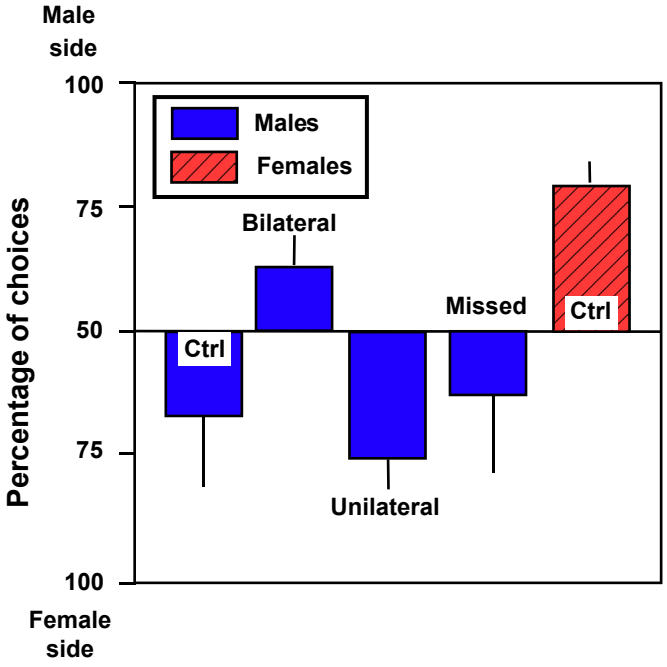

FIGURE 48.4 Effect of lesions of the medial preoptic area on sexual preferences in male ferrets. Control males (Ctrl) spend the majority of their time in the compartment containing a female and vice versa. Male preferences are reversed following a bilateral lesion of the preoptic area but not by unilateral lesions or lesions of a different, usually adjacent, brain site (missed). *Source: Redrawn from data in Ref. 65.*

function such as the appeasement of dominance/subordination relationships.

One instance of spontaneous (not experimentally induced) exclusive same-sex sexual preference that appears very similar to human homosexuality has, however, been detected and studied in some detail. This example concerning populations of domestic male sheep (*Ovis aries*) in Western USA (Idaho) thus deserves to be reviewed in detail.[72]

In this sheep population, a significant fraction of the males (~8%) show little or no reaction in the presence of females, but are nevertheless not asexual—they display active sexual behavior if given another ram as a sexual stimulus. These males even mate exclusively with other males when given a choice between a male or female partner.

Anatomical studies have shown that the sexually dimorphic nucleus of the preoptic area in these sheep differs significantly according to their sexual partner preference: the ovine sexually dimorphic nucleus of the preoptic area (oSDN) is approximately three times larger and contains about four times more neurons in males than in females, but in male-oriented rams (MOR) it is significantly smaller and contains fewer neurons than in female-orientated rams (FOR).[73] The oSDN of MOR also expresses aromatase at a reduced level as compared to FOR. The oSDN of MOR is thus quite similar to the oSDN of females and correlates with sexual orientation—subjects attracted to males (females and MOR) have a smaller oSDN and express less aromatase than subjects attracted to females (FOR).[72,74]

The small size, and other feminized characteristics of the oSDN in MOR, could technically be a consequence of their orientation, since the brain is plastic and responds to the social environment. However, Roselli and colleagues demonstrated that the oSDN is already sexually differentiated during embryonic life (around day 135), presumably under the influence of testosterone in males. Indeed, embryonic treatment of females with testosterone between 30 and 90 days of gestation results in a masculinized oSDN in females.[75] Furthermore, the size of this nucleus is no longer modified in adulthood by manipulations of plasma testosterone concentrations.[75] Therefore, the small size of the oSDN in MOR is presumably determined before birth, and before the subjects had an opportunity to express their sexual orientation. Given that this nucleus is located in the center of the preoptic area, a region involved in the control of sexual orientation, there is every reason to believe that the little oSDN of MOR is (one of) the cause(s) of their atypical sexual orientation resulting from an inadequate masculinization by testosterone during embryonic life.

In conclusion, available animal studies demonstrate that sexual partner preference (sexual orientation) in animals is a sexually differentiated feature exactly like other sexually differentiated behaviors or morphological characteristics. Sexual orientation is controlled at least in part by the preoptic area (like sexual behavior), and it differentiates under the influence of pre/perinatal sex steroids.

Endocrine Control of Sexual Partner Preference in Humans

Since embryonic sex steroids organize the genital morphology in humans as they do in rodents, and probably have similar effects of the organization of the brain and behavior,[76–79] it is legitimate to wonder whether these hormones also have an impact on the development of human sexual orientation. One could indeed imagine that exposure to a high male-typical concentration of testosterone during a critical phase of development would lead to a male-typical orientation (attraction to women), whereas a lower embryonic exposure to steroids would lead to a female-typical orientation (attraction to men; see Figure 48.5). There would be a minimal concentration of testosterone required to masculinize this feature like other aspects of behavior in animals (and humans).

It should also be noted that the critical mechanism here is testosterone action in the brain. This action could be affected by changes in circulating concentrations of the steroid, but also by any modification in the complex cellular processes that translate the presence of the steroid at its target into the activation of a cellular process. Remember that to produce its effects, testosterone must often be metabolized in its target structures (aromatized in the brain, 5α-reduced in the brain and genital area), bind to specific receptors and then activate in conjunction with various steroid receptor co-regulator (co-activators and co-repressors) complex intracellular signaling cascades eventually leading to changes in protein synthesis and/or neural activity.[80–82] Any aspect of this complex suite of actions could be affected in the brain, in the absence of changes in testosterone plasma concentrations, and consequently of testosterone action in the periphery (e.g., on the sexual differentiation of genital structures).

Since the embryonic steroid concentration and action are likely to vary between subjects due to genetic differences, exposure to diverse environmental factors, or specific endocrine pathologies, one could expect that male subjects at the lower end of the distribution could acquire a female-typical orientation (and be gay), whereas females at the high end of the concentration curve would acquire a male-typical sexual attraction (and be lesbian; see Figure 48.5). Even if they are probably not attracted by the same (type of) individuals, in terms of definition of the attractive sex, females and gay men are indeed similar, while males and lesbians share an attraction for women.

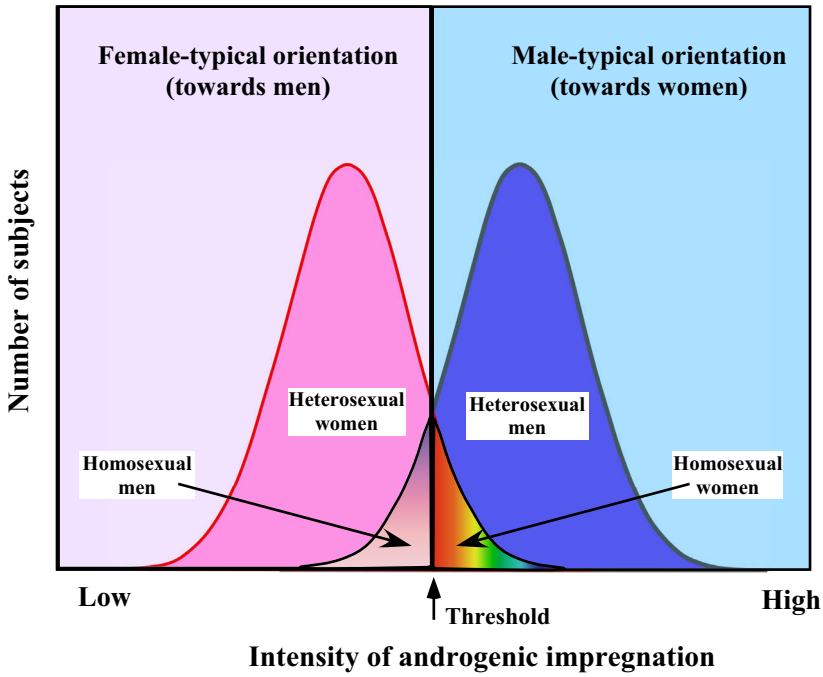

FIGURE 48.5 **Theoretical model illustrating how fluctuations around the average concentration or action of testosterone in male and female embryos in humans or other mammalian species could affect sexual orientation and partner preference.** Male embryos at the low end of the male distribution and female embryos at the high end of the female distribution are, in this model, beyond a critical threshold of testosterone action and thus exposed to a testosterone concentration or action able to produce a phenotype characteristic of the opposite sex. In this condition they would develop a homosexual orientation.

As described in the following sections, two types of evidence actually support this notion; they relate to (1) clinical studies demonstrating that pathologies affecting the embryonic endocrine milieu are associated with increased incidence of homosexuality and (2) the presence of sexually differentiated characteristics that are different in gays and lesbians as compared to corresponding heterosexual populations.

Clinical Studies

There are several clinical conditions in which substantial modifications of the prenatal endocrine environment are clearly documented. It can be hypothesized, based on the animal studies and on the theoretical models that were just reviewed, that these endocrine changes should affect the development of sexual orientation. In a few selected cases, the most interesting ones from a scientific point of view, the embryonic endocrine changes are expected to push sexual orientation in a direction opposite to the effects of the postnatal social/educational environment. These cases therefore provide a useful test of the relative role of both types of factors in the control of sexual orientation. Four such clinical conditions are important to discuss here since they were shown to be associated with a significantly increased incidence of homosexual orientation (in some studies up to 40% compared to less than 10% in control populations).

CONGENITAL ADRENAL HYPERPLASIA

Congenital adrenal hyperplasia (CAH), *formerly called adrenogenital syndrome or congenital virilizing hyperplasia*, is a genetic disease that affects one of the enzymes involved in the synthesis of cortisol (often 17α-or 21-hydroxylase) in the adrenal glands. This indirectly results in the hypersecretion of androgens in the affected embryos.[83,84] These girls exposed in utero to abnormally high levels of androgens show a more or less profound masculinization of genital structures and of a variety of behavioral traits (e.g., aggressive play, type of drawings produced spontaneously, high physical activity and toy selection, occupational interests).[79,83,85–88]

The masculinization of external genital structures in CAH girls is usually detected at birth and corrected surgically to remove part of the penis to reproduce a clitoris and reopen the vaginal opening. Their endocrine milieu is adjusted by administration of exogenous glucocorticoids that will also reduce the inappropriate production of androgens through their feedback action on the brain and pituitary gland to reduce adrenocorticotropic hormone (ACTH) secretion that drives adrenal steroidogenesis, and these genetically XX children are then raised as girls.

Despite being raised as girls, when they become adults CAH women display a markedly increased probability of commitment or desire to engage in a homosexual relationship in comparison to control females or to unaffected sisters[89–91] (see Ref. 79 for a recent review). Some studies reported up to 30–40% of CAH subjects showing some form of homosexual attraction as compared to 10% or less in control populations. Other recent studies, sometimes based on much larger sample sizes, have produced similar figures, although other studies indicated a lower incidence, in the 20% range.[92–94] Overall, only one out of 10 studies failed to find an increased

incidence of homosexuality in CAH women (see Ref. 79). Meyer-Bahlburg and colleagues[94] also showed that the degree of virilization in various forms of CAH correlates with the increased incidence of bi-or homosexuality (Figure 48.6).

Because the endocrine defect was corrected soon after birth, genital structures were surgically feminized, and these girls were presumably raised as girls; these data suggest that prenatal androgens are involved in the determination of sexual orientation in women, although alternative interpretations of these data have been proposed (e.g., suboptimal genital structures despite surgical correction making heterosexual activity difficult or painful; see Ref. 79).

It should also be noted that (1) even if the percentage of women with homosexual tendencies is significantly increased in CAH subjects, this only concerns less than half of the subjects[79] and (2) the reported percentages often concern subjects that display a sexual orientation that is not strictly heterosexual but not necessarily exclusively homosexual. Sexual orientation is frequently quantified on a seven-point ordinal scale, ranging from 0 (strictly heterosexual) to 6 (strictly homosexual), called the Kinsey score. The increased incidence of homosexual tendencies described in CAH subjects often refers to subjects with a Kinsey score greater than 1, but include only rare cases of exclusively homosexual subjects (Kinsey score of 6). Thus if these data strongly suggest a role for prenatal testosterone in the control of sexual orientation, they also clearly indicate that this steroid cannot be the only factor that determines this orientation. Accordingly, the vast majority of lesbians were not affected by CAH during their early life.

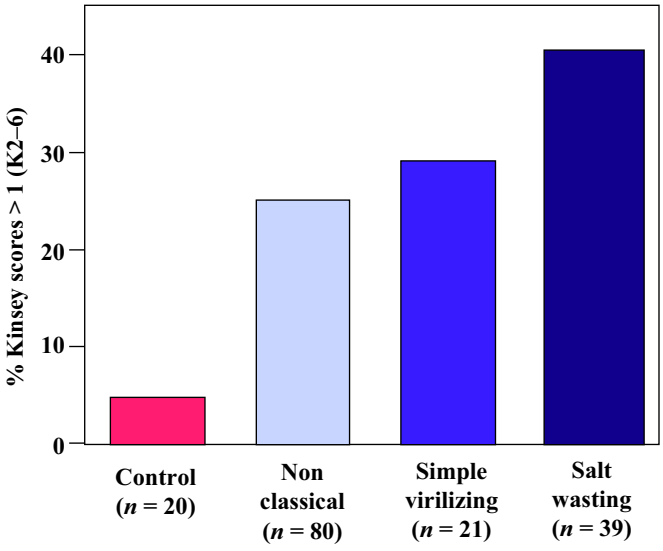

FIGURE 48.6 Percentages of bi- or homosexual subjects expressed as the ratio of the numbers of subjects rating between K2 (largely heterosexual but also distinctly homosexual) and K6 (completely homosexual) on the Kinsey scale to the total number of subjects (K0 to K6) in girls exposed to prenatal androgenization linked to the syndrome of congenital adrenal hyperplasia (CAH). This percentage increases with the severity of the CAH phenotype from the nonclassical (NC) to the simple virilizing (SV) to the salt-wasting (SW) type. The number of subjects in each category of CAH is indicated below the graph. *Source: Drawn from data in Ref. 93.*

TREATMENT OF PREGNANT MOTHERS WITH DIETHYLSTILBESTROL (DES)

Between 1939 and 1960, 1–5 million pregnant women were treated with DES in Europe and the United States to prevent miscarriage. This treatment was not only ineffective but even turned out to have detrimental long-term consequences (e.g., increased incidence of cancer in the vagina and cervix). One of the unexpected consequence of DES exposure was that girls born from these treated mothers showed, when adult, a significant increase in homosexual fantasies or nonheterosexual sexual activity (bi- or homosexual), despite the fact that their rearing had obviously been consistent with their genetic female sex (the change in sexual orientation was not anticipated).

This increased incidence of bi- or homosexuality was confirmed in several independent studies.[95–97] It affected a percentage of subjects ranging from 20% to 40% depending on the sample investigated. The reproducibility of this effect was, however, questioned by a more recent study based on a very large number of subjects,[98] but this study was probably less sensitive and only assessed the actual homosexual activity on a qualitative scale (presence/absence), whereas previous studies also analyzed other aspects of sexuality (fantasies, dreams) and used a graded scale (Kinsey scale in seven points).

If the effect of DES is real, which will be difficult to confirm since this treatment was abandoned a long time ago, this would indicate that estrogens as well as androgens are able to masculinize sexual orientation. This is consistent with rodent data, where many effects of testosterone on sexual differentiation are produced after conversion into estradiol by aromatase in the brain, but would conflict with other human data that tend to assign a prominent role to signaling via androgen receptors in sexual differentiation.

CLOACAL EXSTROPHY

Complex genitourinary malformation occasionally occurs during embryonic development, resulting in the birth of XY males who, in addition to various malformations of the pelvis, have no penis. These subjects have normal testes and were thus presumably exposed to a male-typical pattern of androgen secretions before birth. Historically, these subjects were submitted to vaginoplasty and removal of the testes soon after birth, and they were raised as girls.

A few follow-up studies have demonstrated that in a significant number of cases (usually about half), these subjects, as adults, chose to adopt a male identity and gender role. Information concerning the sexual orientation of these subjects is sparse, but in many cases, a typical male sexual orientation (attraction to women) was also observed that would once again relate to their embryonic exposure to androgens.[99–101]

Importantly, these masculine features developed in the absence of pubertal action of testosterone, since most subjects were castrated soon after birth. Prenatal or immediately postnatal androgens would thus explain the high incidence of changes in gender identity (and probably sexual orientation) observed later in life in these XY subjects, despite the fact that they were subsequently raised as girls. This change, however, did not occur in all individuals. It is thus consistent with a role of prenatal androgens, but these data also indicate that androgens are probably not the only determinant of sexual orientation.

5α -REDUCTASE DEFICIENCY

Testosterone induces the development of male external genitals by fusion of the genital folds to form a scrotum and the development of the genital tubercle in a penis through its conversion into DHT by the enzyme, 5α-reductase. In the absence of DHT, the genital folds do not fuse, and instead form the vaginal lips, while the genital tubercle develops only very little and turns into a clitoris. Imperato-McGinley and colleagues identified, in the Dominican Republic, a mutation of 5α-reductase, which prevents the production of DHT in the genital skin.[102,103] This recessive mutation has also been discovered in Papua New Guinea, another island where inbreeding is prevalent.

Individuals affected by this mutation are born with genital structures that are not masculinized and are (presumably) raised as girls. They provide, therefore, another test of the relative power of the potentially opposite effects of the embryonic endocrine environment (presence of testosterone and also probably estrogens) and rearing on sexual orientation. The rise in plasma testosterone associated with puberty later masculinizes (at least in part) the genital structures, and these individuals usually revert to a male gender and male-typical sexual orientation. This change of identity and gender role has even been observed in some individuals who had been married at a very young age to a man as a girl and who remarried with a woman after puberty. In terms of sexual orientation, these adults usually show a male-typical attraction to women.

It has been argued that the relative ease with which these subjects apparently change gender and sexual orientation at puberty, despite having been raised as females, was related to their exposure to androgens during embryonic life (testosterone secretion is apparently normal in these subjects, it is only its 5α-reduction that is deficient). However, the medical condition of these subjects is often identified at birth, so that their sex of rearing is not necessarily unequivocally female. Furthermore, the obvious social advantages related to adopting a male gender identity in the societies where this 5α-reductase deficiency frequently occurs raises additional questions about the reasons underlying the postpubertal sex change. Note, however, that Imperato-McGinley and colleagues showed that in a group of 18 affected subjects, who had completely female external genitalia at birth and had therefore been brought up in the firm belief that they were girls, 17 adopted a male sexual identity after puberty and were sexually attracted to women.[104]

OTHER EMBRYONIC ENDOCRINE CONDITIONS OF POTENTIAL SIGNIFICANCE

A number of other endocrine perturbations of hormonal secretion or action are known to occur in humans and are similarly associated with significant discrepancies between sexual orientation and genetic sex (see Ref. 97). The role of early hormones in these cases is, however, more difficult to separate from the role of postnatal rearing because these early endocrine disruptions lead to a (nearly) complete sex reversal, so that subjects are raised completely assuming a sex (and gender) that is opposite to their genetic sex. For example, XY subjects with complete androgen insensitivity syndrome are born with female genitalia and are typically raised as girls at least until puberty, when the absence of menstruation leads to medical examination and diagnosis. These subjects usually have a female gender identity and a female-typical sexual orientation (they are sexually attracted to men). This demonstrates that sexual orientation is not necessarily associated with the genetic sex. Their sexual orientation is consistent with their embryonic exposure to steroids (no action of androgens leading to a female-typical orientation) but it is impossible in these cases to discriminate between the role of prenatal hormones and the role of the education and postnatal environment, because both potentially concur to produce a female-typical orientation. The same potential confusion is, to some extent, associated with the 5α-reductase deficiency affecting XY subjects since one cannot be completely sure that they were really raised as girls.

Altogether, these clinical conditions (two in males and two in females) nevertheless support the idea that embryonic hormones play a substantial role in determining adult sexual orientation. It should be noted, however, that these changes in sexual orientation as a result of embryonic endocrine disruption always concern only a fraction of affected individuals (usually a maximum of 30–40% showing a partially reversed orientation) so that

at least 60–70% of subjects exposed to these altered endocrine conditions retain their heterosexual orientation.

Sexually Differentiated Characteristics

The optimal way to directly test the importance of embryonic steroids in the determination of sexual orientation would be to monitor these concentrations in a large number of subjects during their fetal, embryonic, and early postnatal life, and then correlate these data with the sexual orientation of these subjects 20–30 years later. However, practical reasons (risks associated with in utero blood sampling, long duration of the study, small percentage of subjects who will turn out to show a homosexual orientation as adults) make it nearly impossible to obtain this type of data. Researchers have therefore used an alternative strategy based on the use of proxies to indirectly assess the endocrine milieu to which specific subjects were exposed during development.

A number of morphological, physiological, and behavioral characteristics are sexually differentiated in animals and in humans. In many cases, these features are known to differentiate under the irreversible organizing influence of embryonic hormones. Many studies have thus quantified these sexually differentiated features comparatively in homo- and heterosexual populations to test, through these proxies, whether homosexual subjects had potentially been exposed to atypical hormonal conditions during their development. Positive results have been obtained in a substantial number of these studies.

The sexually differentiated characteristics that have been studied in this context include variables that are presumably influenced by prenatal androgens, but could also possibly be secondarily affected by the sexual orientation of the subject. This is namely the case for the performance of adult subjects in various cognitive tests (e.g., Ref. 105) or for the physiological brain responses to odors associated with androgenic or estrogenic steroids produced by males or females.[106,107] These traits are significantly different in homo- and heterosexual men and/or women, but it remains unclear whether these differences reflect a differential prenatal exposure to androgens or an indirect consequence of their sexual orientation resulting, for example, from conditioned responses. These differences will not be reviewed here since they do not represent conclusive evidence for exposure to an atypical prenatal endocrine milieu, but they are discussed in detail elsewhere.[42,43,108]

We will focus our attention here on a few morphological and physiological features that are, on the one hand, clearly influenced by prenatal testosterone and, on another hand, significantly modified in gays or lesbians. These features are also very stable characteristics, so that it is difficult to conceive of how they could possibly be affected secondarily by the adult sexual orientation. They provide, therefore, important evidence suggesting exposure to an atypical endocrine environment in early life. A more extensive presentation of these data is available elsewhere.[42,43,108]

THE RELATIVE LENGTH OF THE INDEX AND RING FINGERS

The ratio of the lengths of the second to the fourth fingers (D2:D4) is significantly smaller in men than in women and in males than in females in a variety of animal species.[109,110] It is masculinized in females of a variety of mammalian and even avian species by injection of androgens during embryonic life.[110–112] In humans, this ratio is masculinized (decreased) in CAH women exposed to an excess of androgens in utero,[113,114] and it is significantly increased in XY subjects affected by the complete androgen insensitivity syndrome.[115] This sex difference in the relative length of fingers in humans thus probably reflects differences in the embryonic androgen concentrations (male > females; see Ref. 116 for review).

Several studies have shown that this ratio is masculinized (smaller) in lesbians as compared to heterosexual women[117–120] (Figure 48.7). Interestingly, one study showed that only lesbians who identify as masculine (known as "butch") had this decreased D2:D4 ratio.[113] Although a failure to replicate this effect has been published[121] and the significance of the D2:D4 ratio has been questioned (e.g., does it reflect differences in bone length or in fat accumulation[122]), the masculinized D2:D4 ratio in lesbians has been confirmed by meta-analyses of

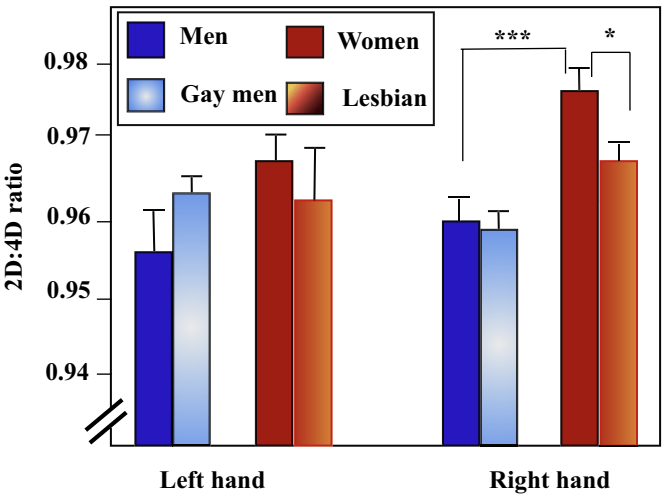

FIGURE 48.7 **Ratio of lengths of the index (2D) and ring finger (4D) as a function of sex and sexual orientation.** This figure is reproduced in color in the color plate section. The 2D:4D ratio is greater in women than in men, probably due to in utero exposure to testosterone of male embryos. It is close to the male level in homosexual women, suggesting that they have been exposed to abnormally high levels of testosterone during a part of their embryonic life. For unknown reasons, these differences are more pronounced in the right hand. *= $p < 0.05$, ***= $p < 0.001$. *Source: Redrawn from Ref. 117.*

available data,[116] suggesting that lesbians, as a group, were exposed early on to higher-than-typical concentrations of androgens.

In contrast, no consistent pattern of changes of this D2:D4 ratio has been so far detected in gay men. The D2:D4 ratio in homosexual men was shown to be lower than (more masculine, opposite to the prediction),[118,119] identical to,[117] or even higher[121] than the ratio in heterosexual men. Thus, only one of these four studies focusing on D2:D4 ratio, and sexual orientation in men is in agreement with the predictions of the prenatal hormonal theory.

THE RELATIVE LENGTH OF LONG BONES

Like the finger bones discussed in the previous section, the length of the long bones of the arm and leg are sexually differentiated and are thought to reflect the influence of the sex-specific embryonic hormonal environment. The length of bones that become sexually differentiated during infancy was shown to be significantly different between homo- and heterosexual individuals, while the bones that become different between men and women after puberty were not modified in a manner associated with sexual orientation.[123] Subjects attracted to men (heterosexual women and homosexual men) have a smaller growth of the bones of the arms, legs, and hands than subjects who have a sexual preference for women (heterosexual men and homosexual women). These differences were particularly noticeable in the analysis of the ratio of the hand width to hand length (see Figure 48.8).

These conclusions are based on the study of nearly 400 subjects, and although they have not been replicated to date, a few more anecdotal studies had already identified, in males, differences in ratios of the lengths of various parts of the body associated with sexual orientation (see Ref. 123). These data support the notion that androgen concentrations or actions were reduced in gay men during development and, conversely, more prominent in homosexual than in heterosexual women.

OTO-ACOUSTIC EMISSIONS

The inner ear, in addition to its obvious function in hearing, also emits sounds in the form of barely audible clicks, called oto-acoustic emissions (OAE). OAE are produced either spontaneously or in response to short noises in the environment (e.g., clicks). These OAE are significantly more frequent and have a larger amplitude in women than in men, and the differences are already present during childhood.[124] These sex differences in OAE have been reported in a variety of animal species, including sheep and monkeys. Most interestingly, in sheep, OAE are masculinized (they decrease in frequency and amplitude) following embryonic treatment of females with androgens, and they are no longer affected by castration in adulthood, indicating that they presumably reflect organizing effects of steroids during early ontogeny.[125]

OAE were shown to be significantly less frequent, and have a lower amplitude, in lesbians as compared to heterosexual women, again suggesting that lesbians have been exposed to higher concentrations of androgens than usual during early life[124,126] (Figure 48.9). In addition, women who had a twin brother and, therefore, presumably had been exposed to slightly higher levels of testosterone during embryonic life (testosterone

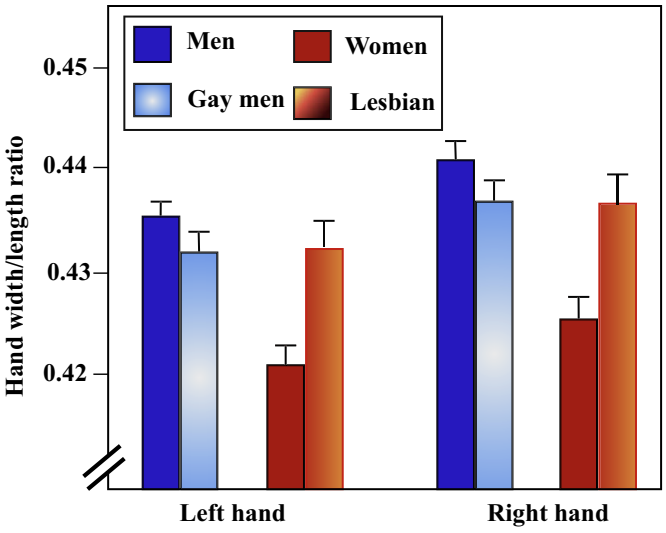

FIGURE 48.8 **Relationship between sex and sexual orientation and the ratio of width to length of the hand.** This figure is reproduced in color in the color plate section. *Source: Redrawn from data in Ref. 123.*

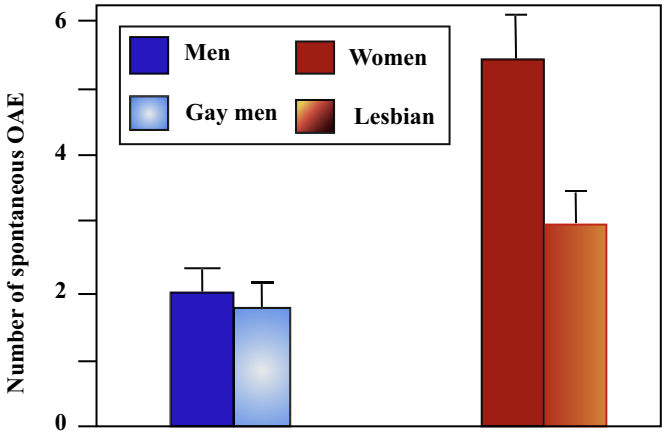

FIGURE 48.9 **Frequency of oto-acoustic emissions (OAE) evoked by click sounds measured in the right ear in men and women as a function of sexual orientation.** This figure is reproduced in color in the color plate section. OAE are sexually differentiated and significantly masculinized in homosexual females. *Source: Redrawn from data in Refs 124,127.*

produced by the twin would have diffused to the female embryo), had masculinized OAE, confirming sensitivity to androgens of the response in humans.[124]

These data are thus consistent with the hormonal theory of sexual orientation, stating that female fetuses destined to become homosexual or bisexual have been exposed to abnormally high levels of androgens. However, contrary to the simplest predictions that could be drawn from this theory, no difference in the OAE has been found between homo- and heterosexual men.

Similar studies have also identified a partial masculinization of evoked acoustic potentials (electrical responses induced in the brain by short auditory stimuli) in homo- or bisexual women compared to heterosexual women.[128] An independent study also demonstrated masculinization in lesbians of the "startle response",[129] another sexually differentiated response (women < men) by which the blink of the eyes following a loud noise is partially inhibited if the loud noise is preceded by a lower noise. In both cases, however, homosexual men failed to show a lack of masculinization of these, or even displayed a hypermasculinization, which disagrees with the hormonal theory of homosexuality in men.

THE POSITIVE FEEDBACK RESPONSE OF LUTEINIZING HORMONE TO ESTROGENS

During the ovulatory cycle in females, plasma estrogen concentrations progressively increase during the follicular phase of the cycle to reach a critical threshold that causes by positive feedback the release of a large peak of luteinizing hormone (LH), ultimately inducing ovulation. In rodents, this positive feedback is observed in females, but not in males, and this sex difference results from the organizing action of sex steroids during the perinatal life (see Chapters 26-28 for a detailed treatment of this topic in rodents, sheep, and primates, respectively).

Dörner originally demonstrated the existence of a differential feedback to estrogens, as measured by plasma LH concentrations, between homo- and heterosexual men.[130] In this study, gay men reacted to an injection of estrogen with a significant increase in blood levels of LH that was lower than in women, but still significantly higher than in heterosexual men.[131–133] For various scientific, but also sociological and ethical reasons whose description goes beyond the present review (see namely Ref. 134), this work raised a huge controversy and was received with skepticism. This differential neuroendocrine reaction to an injection of exogenous estrogens was, however, reproduced in an independent study performed in the United States. Gladue and colleagues demonstrated that after a single injection of Premarin, a mixture of compounds with estrogenic action, women showed 72–96 h later a major increase in LH plasma concentrations.[135] Such an increase was not observed in men, but again an increase of intermediate magnitude was observed in homosexual men (see Figure 48.10).

As noted previously, this increase in LH concentration in humans does not reproduce the preovulatory peak observed in female rats (see for discussion Ref. 134) because it is clearly delayed and less abrupt. It must also be stressed that the sex difference in positive feedback of estrogens on LH that is clearly observed in rats but also in mouse, hamster, and guinea pig, and results from the masculinization/defeminization of males by androgens during the perinatal period, is not present in rhesus monkeys[136] and thus also probably not in humans. This conclusion is, however, difficult to formally confirm in our own species since in order to compare the positive feedback response to estrogens in both sexes it is necessary to place all subjects (males and females) in a similar endocrine condition, which cannot be done in humans for obvious ethical reasons.

It is thus impossible to determine whether the difference observed between homo- and heterosexual men concerns a neuroendocrine mechanism that differentiates (is defeminized) under the influence of gonadal steroids during early life, as observed in rats,[137] or more likely reflects another type of difference (e.g., in testicular or pituitary physiology and thus in basal endocrine condition) that could interfere with the positive and negative feedback.[134,138] In any case, it appears that male homosexuality is associated with a difference in functioning

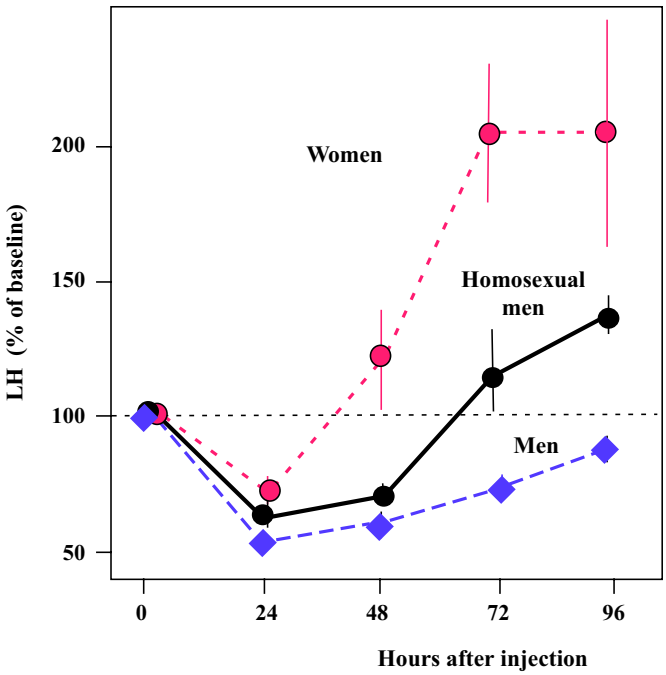

FIGURE 48.10 **Changes in blood concentrations of luteinizing hormone (LH) in response to injection of a single dose of the estrogenic compound Premarin in heterosexual men and women and in homosexual men.** *Source: Redrawn from Ref. 135.*

of the hypothalamic-pituitary-testicular axis that is very unlikely to be the result of a personal choice.

BRAIN STRUCTURES

Several sexually differentiated brain structures were also shown to be different between homo- and heterosexual subjects, although these studies have focused almost exclusively on males. Historically, the first identified brain difference concerned the suprachiasmatic nucleus, the central clock of the organism, that was shown to be significantly larger (1.7X) and contains twice as many neurons in gay men than in heterosexual subjects.[139] This nucleus is, however, not known to be sexually differentiated in control heterosexual populations, and its links to reproduction are only indirect. The significance of this difference and its potential relationship with sexual orientation is thus difficult to assess.

It was subsequently reported that the size of the anterior commissure, when measured in the mid-sagittal plane, is larger in gay men than in heterosexual control subjects.[140] This difference is more interesting for the purposes of the present discussion because the size of this commissure is known to be larger in women than in men. However the size of this commissure has no obvious relationship with sexual orientation (it could relate to functional lateralization), so that the meaning of the difference between homo- and heterosexual men remains difficult to interpret.

Gorski and colleagues (who had identified the SDN-POA of rats) also discovered in the human preoptic area four cellular condensations that they labeled interstitial nuclei of the anterior hypothalamus (INAH) 1 to 4. Two of these nuclei (INAH 2 and 3) are significantly larger in men than in women.[63] Subsequent studies showed that INAH3 is significantly smaller in homosexual men than heterosexual men, so that its size is essentially equal to what is observed in women[141] (Figure 48.11).

An independent study based on different brains confirmed the reduced size of INAH3 in male homosexuals compared to heterosexuals, though the magnitude of the difference observed in this replication was lower than in the original study and not statistically significant.[142] In this study homosexual men had a greater cell density (more cells per unit volume), but a similar total number of neurons in INAH3, when compared to heterosexual subjects—neurons were more densely packed potentially because they formed fewer synapses (during development?).

The mechanism(s) that control(s) the development of this nucleus in humans is unknown, but it does not seem to depend significantly on hormonal status in adulthood.[143] In rats and sheep, the size of a potentially homologous nucleus located in the same part of the preoptic area (SDN-POA in rat, oSDN in sheep) is irreversibly determined by the action of embryonic sex steroids.[68,75,144,145] Moreover, lesions of this nucleus in adult rats or ferrets modify male sexual partner preference, which can be considered an animal model of sexual orientation.[65,66]

If the same holds true in humans, the smaller INAH3 of gay men could then be a marker of deficient exposure to androgens during early ontogeny, and even could be a cause of the modified sexual orientation. There are, however, multiple considerations that severely limit this conclusion, the most important being that it has been, so far, impossible to determine whether the small INAH3 of gay men is a cause or consequence of their sexual orientation (see Ref. 141 for discussion). Based on analogies/homologies with animal models (see namely Refs 53,54,69) and on studies of the oSDN in ram-oriented

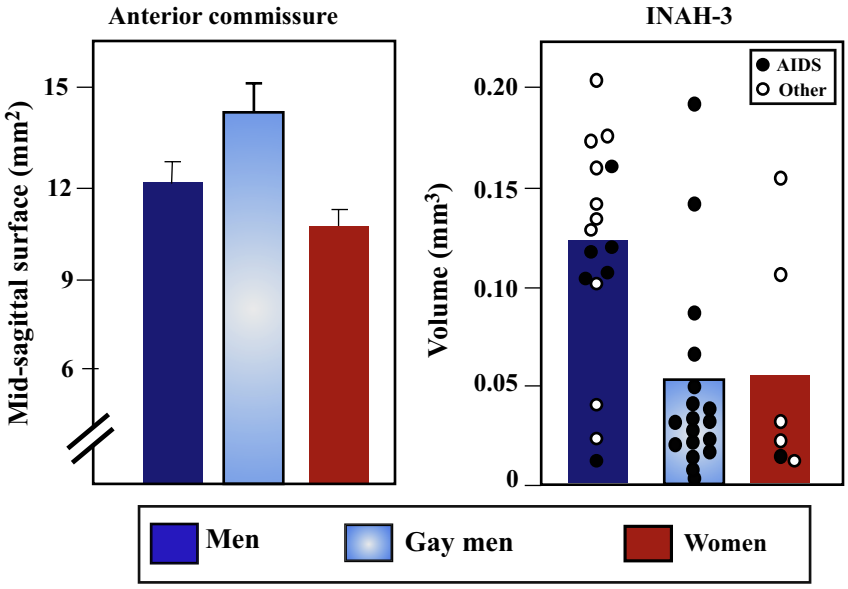

FIGURE 48.11 Average size of the anterior commissure as estimated by its surface in the mid-sagittal plane (left) and volume of the 3rd interstitial nucleus of the anterior hypothalamus (INA-3) (right) in men, gay men, and women. This figure is reproduced in color in the color plate section. Data for the anterior commissure are presented as means ± SEM, whereas for INAH-3 the average value in each group, as well as all individual data points, are shown to clearly illustrate the partial overlap between groups despite the significant differences between men and women and between men and gay men. Individual data points are filled in subjects who died from AIDS and open in subjects who died from other causes. The smaller average size of INAH-3 in gay men cannot be accounted for by the fact that all subjects included in the study had died from AIDS since in the control group, the INAH-3 of men who died from AIDS is not smaller than in men who died from other causes. *Source: Redrawn from data in Refs 140,141.*

male sheep,[73,75,146] we are, however, tempted to favor the first of these interpretations. The small INAH3 of homosexuals should, at the very minimum, represent the signature of an atypical embryonic hormonal environment (see Refs 43,108 for a detailed discussion).

Conclusions Concerning the Endocrine Control of Sexual Orientation

Taken together, these clinical studies and the analyses of phenotypic differences between homo- and heterosexual populations strongly support the notion that prenatal steroids, in particular testosterone, play a significant role in the development of adult sexual orientation in humans. Several aspects of these results clearly indicate, however, that this early testosterone action is not sufficient in itself to determine this orientation. On the one hand, clinical studies of subjects that were exposed to atypical endocrine conditions for their sex reveal an increased incidence of homosexual subjects, but this incidence never reaches more than 30–40%, which means that the majority of subjects (60–70%) remain heterosexual even after exposure to these atypical endocrine conditions. On the other hand, the sexually differentiated features that distinguish homo- and heterosexual populations do so in a statistical manner, but there is always a large degree of overlap between these features in the two populations. Furthermore, these phenotypic differences are often seen in one sex but not the other. For example, the masculinization of the D2:D4 finger ratio and of the frequency of OAE is observed in lesbians, but the opposite effect (lack of masculinization) has not been reliably detected in gay men as would be predicted by the simplest form of the endocrine theory of sexual orientation. Conversely, a lack of masculinization has been observed in the length of long bones and in the volume of the INAH3 of homosexual men, but the corresponding studies have not been performed in women, and it is thus unknown whether these features are masculinized in lesbians.

Multiple theoretical reasons identified in animal studies are available to potentially explain the limitations described herein. They are based on the existence of critical sensitive periods of testosterone action, on thresholds in the dose–response curves linking testosterone to its phenotypic effects, but also on probable differences between homosexual populations from different studies based on the sampling methods that were used (global analysis of college populations, recruitment in gay bars or in gay pride parades, etc.). It is also likely that homosexual populations are not homogenous (see, for example, the recognized differences between "butch" and "femme" lesbians) and testosterone might be implicated in the development of some, but not other forms of homosexuality. At this time, results are not available to discriminate between these different interpretations and to answer the question of the relative importance of the embryonic endocrine environment versus other factors in the control of sexual orientation. It can just be said that these hormones have a definite impact.

Changes in Hormone Action during Prenatal Life?

Two major questions are raised by this endocrine theory of sexual orientation: (1) how can sexual orientation be modified without changing other hormone-dependent sexually differentiated characteristics, such as genital structures, that do not differ between homo- and heterosexual subjects, and (2) what is the possible origin of the changes in hormone secretion or action that putatively induce a homosexual orientation?

The discordance between genital structures and sexual orientation can be understood in the context of an endocrine model if one assumes that these two steroid-dependent responses develop at different times (this is the case since the genitals are differentiated early during embryonic life when the brain is still poorly developed), or that they are associated with different dose responses (no clear data on this topic in humans) so that a change in hormonal environment will affect one but not the other, or finally, if the endocrine factor controlling the development of homosexuality is not the circulating level of the steroid (testosterone), but rather one aspect of its action in a specific part of the brain (change in local concentration of receptors, in expression of a steroid receptor co-activator, etc.). It is therefore easy, in theory, to reconcile an endocrine theory of homosexuality with the presence of sex-typical genital structure. The origins of the endocrine changes leading to homosexual orientation are in contrast less clear.

Studies in rats showed that stressing pregnant females partly blocks the masculinization of their male fetuses so that they display a decreased anogenital distance at birth, they mount females less frequently, and are even capable of presenting female-typical behaviors such as lordosis.[147] The volume of their SDN-POA is also smaller than in control males.[148,149] Dörner showed, through a retrospective analysis, the existence of a significant peak in frequency of gay men among cohorts of boys born in Berlin between 1942 and 1946, a period when mothers obviously experienced an unusually high level of stress.[133,150,151] There was also a positive correlation between stressful life events remembered by mothers and the likelihood that their boy born in this period would be homosexual.[151]

These studies have, however, been criticized on methodological grounds, and recent studies failed to reproduce this correlation between maternal stress and homosexual behavior[152,153] or produced only equivocal

data.[154] Thus the contribution of prenatal stress to human sexual orientation remain elusive.

Interestingly, recent work in rodents has indicated that the masculinizing effects of neonatal estrogens on sexual behavior and aspects of brain structure are often mediated at the cellular level by intracellular changes in the concentrations of prostaglandins (in particular PGE2) resulting from an increase in the activity of their synthesizing enzyme, the cyclo-oxygenase enzymes COX-1 and COX-2[155] (reviewed in Ref. 156). Prostaglandins are lipid signaling molecules that subserve cellular functions related to reproduction, but also inflammation, hyperalgesia, and fever.[157] It is conceivable that their increase during an episode of prenatal inflammation, or the blockade of their synthesis following ingestion of a COX-2 inhibitor such as indomethacin (a commonly used anti-inflammatory drug), could interfere with the normal differentiating effects of sex steroids on the brain and thus alter the development of sexual orientation. One would predict that exposure to indomethacin would block aspects of brain masculinization, resulting in male homosexuality, while an increased masculinization would follow an inflammatory episode and the associated increase in PEG2 resulting in female homosexuality. However, this potential mechanism has not been investigated to our knowledge.

It is also important to emphasize here that a huge amount of data has accumulated during the last few decades indicating that a variety of chemical compounds released in the environment, the "endocrine disruptors," affect endocrine physiology and, in particular, sexual differentiation in many animal species and possibly also in humans.[158–160] No study has, however, to our knowledge, related this exposure to environmental endocrine disruptors to changes in sexual orientation in humans, but this possibility deserves to be investigated.[159]

Genetic Influences

The Heritability of Sexual Orientation

An alternative explanation of the differences in embryonic steroid concentration or action potentially responsible for differences in sexual orientation is genetics. Numerous epidemiological studies have indeed demonstrated the presence of a significant degree of heritability of sexual orientation. Statistically, the concordance of sexual orientation is directly correlated with genetic relatedness. For example, if, in a given population, a son is gay, 20–25% of his brothers will share the same orientation, as compared to 4–6% in a control population.[105,161] Similarly, lesbians have a greater probability than heterosexual women of having a homosexual sister.

Twins studies strongly suggest that this correspondence in sexual orientation does not reflect a communality of postnatal experiences (psychosocial factors), but rather genetic similarity. There is indeed, a much better agreement of sexual orientation in monozygotic (identical) twins than in dizygotic (fraternal) twins born from different ova and sperm. A literature survey[161] showed that, on average, if a dizygotic gay twin has a brother, there is a 15% probability that the brother will also be homosexual, but this probability rises to 65% in monozygotic twins. Another study in the 1990s identified a similar correlation between genetic relatedness and concordance in sexual orientation (monozygotic twins: 52%, dizygotic twins: 22%, and adoptive brothers: 11%; see Ref. 162). Two more recent studies,[163,164] based on large populations of twins and a rigorous selection of subjects, reported, however, a lower concordance between monozygotic twins (20–30%), and a concordance between sexual orientation of twins was also detected in females (0.58, heritability[165]; see Ref. 105 for more detail).

Overall, these studies suggest that, in social conditions typical of Western societies, 30–60% of the variance in sexual orientation in humans should have a genetic origin.[97,105,166] The mechanism supporting this transmission has, however, not been identified to date despite relatively intensive research. Two experimental approaches were used in this context: a candidate gene approach and a genome-wide screening based on linkage analysis.

Candidate Gene Approach

Genetic differences could affect the synthesis of steroid hormones, or their activity, in the brain of the fetus. It is obvious that genes coding for androgen or estrogen receptors, or for aromatase, cannot be completely disrupted in homosexual subjects because these subjects would then suffer from multiple physiological and morphological deficits. Smaller variations (mutation or polymorphism) in these genes might, however, slightly alter the function of the corresponding receptors or enzymes, and in this way modify brain differentiation specifically without affecting peripheral morphology and physiology. Several studies have investigated this possibility in relation to the androgen receptor, and to aromatase, but to date none of them has been able to identify variations in these genes that are significantly linked to sexual orientation.[167,168]

"Direct" versus "Indirect" Genetic Effects

Historically, it has been accepted since the pioneering work of Jost,[169] and the seminal publications of Young and colleagues extending the concept to behavior,[46] that most if not all sex differences in morphology, physiology, and behavior are due to either a differential activation in adulthood by sex steroids or an irreversible organizing action exerted by these steroids during early life. The discovery of a few "apparent" exceptions to this rule has raised doubts about the generality of this rule. It was, for example, discovered that the scrotum in marsupials

develops before gonadal differentiation[170] and that the sexual differentiation of the song control system of zebra finches (*T. guttata*) does not react in the expected manner to pharmacological manipulations of early steroid action.[171]

During the last decade, genetic manipulations have allowed the development of a mouse model in which effects of the genetic sex (presence of XX or XY chromosomes) can be differentiated from the hormonal influence of the embryonic gonad (testes secreting testosterone or relatively inactive ovaries). In some of these mice, the SRY gene that determines the development of the testes has been inactivated by a mutation, whereas in other subjects this same gene has been added to an autosome such that XX genetic females can have a functional SRY gene and thus develop testes and be exposed to the hormonal action of androgens during embryonic development. This "four core genotypes" model thus discriminates on the one hand between subjects that have testes during development (XY and XX^{sry} subjects) and those who don't (XX and XY^{sry-}), and on the other hand between subjects that possess two X (XX and XX^{sry}) or an X and a Y (XY and XY^{sry-}) chromosomes.[172]

The review of data obtained with this model is beyond the goals of the present chapter (see Refs 173,174 for recent reviews), but it is critical to mention that the four core genotypes have revealed that multiple physiological or brain sex differences do not develop under the influence of embryonic testicular secretions but rather relate to the chromosomal complement of the subject. It is thus conceivable that individual differences in sexual orientation could relate to such "direct" genetic effects, i.e., to effects that would not be mediated through a change in steroid action during development.

Genome-Wide Screening

During the search for relevant genes, it was shown that sexual orientation in men tends to be transmitted through matriarchal lineage: a gay man has a higher probability of having gay men among his ancestors on the maternal side (uncles, cousins), but not the paternal side. This was originally interpreted as a sign of inheritance through gene(s) located on the X chromosome, and one study indeed identified a linkage with markers located in the subtelomeric region of the long arm of the X chromosome, a region called Xq28.[175] This association with Xq28 was replicated in two subsequent studies[176,177] but not in a fourth one.[178]

A comprehensive analysis of all available data, including negative results, however, indicates that approximately 64% of gay brothers have common alleles in the Xq28 region of chromosome X instead of the expected 50%,[179] and that this association is highly significant ($p < 0.0001$). However, this chromosomal region remains quite large and could contain many genes. Those that could potentially be responsible for the predisposition to a homosexual orientation have not been identified.[179,180]

In women, similar studies have also detected an increased rate of "nonheterosexuality" (sum of homo- and bisexuality) in nieces and cousins of the paternal lineage of lesbians. This transmission is also consistent with a link to the X chromosome, but it could come from the father as well as the mother. Other interpretations are also possible, and the interpretation of these data remains difficult.[105] Surprisingly, however, the gene(s) located on the X chromosomal regions that could predispose to homosexuality has (have) still not been identified.

Epigenetics

Because molecular studies have in general failed to identify a gene, or groups of genes related to sexual orientation, recent work has focused on the idea that the heritage of sexual orientation could be the result of epigenetic modifications of gene expression. The relevant genes could in this theory be located on sex chromosomes as well as other chromosomes (autosomes).

Studies have in particular focused on the phenomenon of X chromosome gene inactivation. Male cells indeed contain a single copy of the X chromosome, while female cells contain two. In mammals, each female cell randomly inactivates one X chromosome during ontogeny to avoid producing a double amount of proteins whose genes are located on the X chromosome. Bockland and collaborators showed that this inactivation is extremely asymmetrical (i.e., one of the X chromosomes inactivated in more than 90% of the cells instead of 50% predicted from random inactivation) more frequently in mothers of homosexual boys (13 of 97 = 13%) than in mothers of heterosexual sons (4 of 103 = 4%), and even more frequently in mothers of two or more gay boys (10/44 = 23%).[181] The specific link between this unusual pattern of X chromosome inactivation and the homosexual orientation of the sons is unclear at this stage. The unusual X inactivation could have a direct influence on the sexual orientation of sons, but it could also be only an indirect consequence of some unknown genetic mechanism influencing sexual orientation. However, these data clearly show that the homosexual orientation of sons correlates with a biological marker in the mothers, further supporting the notion that sexual orientation is influenced or determined by biological factors. These findings also support a role of the X chromosome in regulating sexual orientation.

A genome-wide linkage study that was not limited, like studies of chromosome X, to subjects with proven maternal transmission, identified a linkage of sexual orientation to loci on chromosome 7 (in 7q36) and 8 (in 8p12), with roughly equal maternal and paternal origin.[182] A marker of specific maternal origin was also found on chromosome 10 (position 10q26). This site with exclusive maternal heritability provides an alternative interpretation to

the transmission of homosexuality through the maternal line. Transmission could be through a gene on the X chromosome, as previously thought,[175] but alternatively, it could reflect maternal transmission of an epigenetic marker on chromosome 10 (see Refs 179,180 for details).

A recent theoretical paper of Rice and colleagues has explored how sex-specific epigenetic markers (epi-marks) could theoretically explain the heritability of sexual orientation in the absence of any identified genetic difference between homo- and heterosexual populations.[82] These authors noted that, although males have on average higher testosterone plasma concentrations than females during embryonic and early postnatal life, this sex difference in circulating concentrations of testosterone has a limited amplitude associated with a large interindividual variance. This is true in both rats and humans, and, furthermore, the sex difference in plasma testosterone concentrations is statistically significant only during short periods of time during development (with often a residual overlap between male and female values). As a consequence, Rice et al. argue that it is difficult to imagine how these limited sex differences in plasma testosterone could by themselves explain the very reliable sex differences in genital structures (sex-atypical genitalia are extremely rare) and even sexual orientation (only a maximum of 10% of subjects have a sex-atypical orientation). Experimental treatment with sex steroids or hormone antagonists reliably modify these characteristics, but the magnitude of endocrine changes that they induce is much larger than the physiological sex differences in circulating steroid concentrations.

Rice and colleagues therefore argued that male and female embryos must have an intrinsic difference in sensitivity to steroid action (hypersensitivity in males and/or hyposensitivity in females) in order to generate the sexually differentiated phenotypes in response to circulating concentrations of testosterone that are only marginally different.[82] This differential sensitivity could relate to sex differences in plasma steroid-binding proteins, in steroid-metabolizing enzymes, in steroid receptors, in steroid receptor co-regulators (activators or inhibitors), or in any subsequent step in the intracellular actions of steroids. Mechanistically, the differences in sensitivity to steroid action could be related to the fact that expression of many genes is already differentiated well before the differentiation of the gonad, at least in the mouse embryo.[183] In the absence of identified differences in gene structure between homo- and heterosexual populations, they propose that sexually antagonistic epigenetic differences transmitted between generations in a sex-specific manner could explain the differential expression of genes that control steroid action in a critical manner. There is actually substantial evidence indicating that already at the blastula stage in cattle, and on day 10.5 postcoitum in mice, which is far in advance of androgen production, there are widespread sex differences in the expression level of hundreds of genes, most of which are autosomal.[184,185] Recent research supports the existence of an early period during development when epigenetic programming of the germ line occurs and permits transmission of aspects of the phenotype to subsequent generations.[186] Rice et al. also indicate, by a mathematical model, that this transmission of sexually antagonistic epi-marks is likely, and would result in the selection and generalization of the relevant epi-marks.[82]

This model seems compatible with all currently available data, and could clearly explain why specific gene(s) implicated in the development of homosexuality have not been identified so far despite the fact that the heritability of this process is clearly established. Whether or not (epi-) genetic mechanisms affect sexual orientation by modifying steroid action during ontogeny or in a more direct manner has not been determined.

The Older Brothers Effect

Finally, to complete the picture on biological factors affecting sexual orientation, we must mention the factor most reliably associated with homosexuality in males, namely, the presence of older brothers born in a family. The incidence of homosexuality increases by 33% for each older brother born to the same mother and is accompanied by a small, but statistically significant, decrease in weight at birth. These effects do not appear to be explained by differences in education or family background (brothers born after the focal subjects, numbers of sisters born to the same mother before or after, whether brothers live together or not, and age of the mother or father are not important) and would be the result of accumulation in the mother, during successive pregnancies, of antibodies against one or more proteins expressed specifically by the male brain. The specific mechanism underlying this phenomenon has not yet been identified, although candidate Y-linked proteins, such as those encoded by genes SMCY, PCDH11Y (protocadherin 11 Y-linked), NLGN4Y (neuroligin 4 Y-linked), and TBL1Y genes have been suggested as potential target(s) for this immune reaction.[187,188]

Interactions between Biological (Hormones, Genes) and Social Factors

Despite their limitations, the endocrine and genetic theories of sexual orientation provide the best explanations to this aspect of mate selection to date. Alternative explanations of homosexuality that are based on psychoanalytical, psychological, or sociological analyses of postnatal social factors may be attractive at first glance and are actually broadly accepted in some societies, have received little, if any, experimental support.[189–191]

These negative results do not necessarily imply a complete lack of influence of the postnatal environment on the development of sexual orientation. This orientation only becomes apparent after initiation of puberty, and there are thus plenty of opportunities for postnatal experiences to influence this phenotypic characteristic. More research is definitely warranted on this topic. It is clear that even if the prenatal factors explain a significant part of the variance and create important predispositions toward homo- or heterosexuality, they do not seem to fully explain sexual orientation, unless one assumes that several of the currently identified prenatal factors must interact to fully determine the adult phenotype.

Alternatively, the postnatal educational or social environment could interact with prenatal biological factors (hormones, genes) to determine adult sexual orientation. Little experimental work on this topic has been made in animal models, but in one species of songbirds, the zebra finch (*T. guttata*), which is known to form long-term sociosexual bonds, research in the Adkins-Regan laboratory has clearly demonstrated that young females treated with estrogens during the first 2 weeks post-hatch will prefer other females over males in two-choice proximity tests, and will pair with females in mixed-sex aviaries where they have ample choice of partners[192–194] (see Refs 193,195 for discussion of a few puzzling aspects of this research, including the fact that unexpectedly treatment with an aromatase inhibitor produces a similar change in partner preference).

However, this reversal of partner preference only develops if the estrogen-treated females are housed in mono-sex female groups during their development. Neither all-female housing alone nor estrogen treatment alone produces a significant modification of the sexual partner preference. The heterosexual partner preference also cannot be modified by steroid treatments in adulthood.

These findings raise the obvious question of what is actually modified by the early estrogen treatment. It has been speculated that the steroid masculinizes the way in which females learn (or become imprinted on) the sex of their partner, but additional work would be needed to resolve this question.

Similar evidence is also emerging in humans suggesting that pre- and postnatal factors could contribute to the control of sexual orientation. Children learn during their first few years that they are male or female and model some of their choices (for a fruit for example) to choices of individuals of the same sex.[196] Recent research suggests that CAH girls exposed prenatally to abnormally high concentrations of androgens are differentially sensitive to these social influences and, in particular, are less prone to copy the behavior of a human model of their own sex.[196,197] These data suggest important new ways in which the prenatal hormonal environment could affect sexual orientation by changing reactions to postnatal social interactions.

Sensory Modalities

Little research has been devoted specifically to the question of the specific signals that are used to distinguish the sex of a potential sex partner. In many mammals, including rodents and possibly humans, olfactory signals seem to play a key role. We already mentioned that cage bedding soiled by rats of the opposite sex induces brain activation, as measured by enhanced expression of the *c-fos* gene, in the olfactory circuits and in areas implicated in the control of sexual behavior.[198,199] This activation was sex reversed in males treated neonatally with an aromatase inhibitor (brain activation after exposure to male rather than female odors[57]).

Similar data have been obtained in humans by imaging techniques such as fMRI or positron emission tomography (PET). The preoptic-hypothalamic area is activated by the odor of specific sex steroids (sometimes considered as putative pheromones, although this idea is far from being universally accepted) of the opposite sex in heterosexual subjects,[200] but these activations are largely sex reversed in homosexual subjects (activation by same sex odors).[106,107] These data were, however, obtained by presenting subjects with concentrations of the steroids that are much higher than in physiological conditions, and the physiological relevance of these results has thus been questioned. A recent study actually indicates that the sex difference in the fMRI response to these odors seems to depend on their concentration.[201] It should also be noted that these activations essentially concern regions of the brain stem and limbic systems that have no or few connections to the telencephalic structures supporting consciousness. There is therefore no indication that these brain activations relate to a conscious selection of the sex of the mating partner.

Other sensory modalities (vision, audition) obviously allow discriminating between males and females in most species, including humans, but to our knowledge, their specific role in sex recognition, independently from recognition of the species or specific individual, is not clearly established.

SPECIFIC INDIVIDUAL FEATURES INFLUENCING MATE CHOICE

Introduction

Once the proper species and sex have been identified, males and females of most species will still not mate indiscriminately. Female fishes in many species will lay their eggs in the open ocean, and males will fertilize

them without making any selection or contact with the female, but in many species, especially those relying on internal fertilization, the specific mating partner will often be selected very carefully.

In ultimate terms, the differential fertility and differential parental investment of males and females has prompted the evolution of different reproductive strategies and leads males and females to select their mate based on different characteristics in order to maximize the survival of their gene pool in the following generations. As mentioned at the outset of this chapter, females who invest large amounts of energy and resources in their reproduction will try to mate with a male in the best possible condition and possessing large amounts of resources (e.g., a large feeding territory) who can not only provide optimal help in raising the offspring (when parental care is present; this is called the direct benefit[3]) but who will also transmit "good" genes to the offspring and thus enhance their survival (indirect benefit). Males in contrast are able to fertilize many eggs and can in many species reproduce at a very low energetic cost. They will therefore pay special attention to the fertility of the females and often try to mate with as many of them as possible. These evolutionary constraints are at the basis of sexual selection and of the polygynous mating system observed in so many species. Sexual selection has thus prompted the evolution and selection during mate choice of secondary sexual characteristics that demonstrate the physical or genetic qualities of the males but the fertility of females.

Phenotypic Traits as Markers of (Genetic) Quality in Males

In order to identify the best possible mate, females rely on indirect markers of genetic quality. A huge amount of ethological research has been carried out, largely in birds and to a lesser extent in mammals, to identify the traits that females are paying attention to when selecting a male partner.[202] These traits are extremely variable. They include morphological as well as behavioral features, and they obviously vary from species to species, so that it would be impossible, and beyond the scope of this chapter, to review them in detail. Morphological characteristics include, for example, the general body size (muscular mass), the color of the plumage and/or its reflectance in ultraviolet light in birds, the development of secondary sexual characteristics such as horns or antlers, wattles and comb in chickens, the color of the penis and testis in some monkey species, and body odors or pheromones[12,203,204] (see Ref. 205 for a detailed presentation and the following section on Optimal Outbreeding).

At the behavioral level, it has been shown in multiple experiments on birds that the complexity of the song repertoire, the length of the song bouts produced by males, the number of different syllables they incorporate, and the amount of songs produced markedly influence female choice.[206–209] The song repertoire has been specifically shown to represent an honest signal predicting the future parental (paternal) investment of a male bird[206] (see Ref. 205).

In humans and other animals, the symmetry of the body is thought to reflect the genetic quality of a subject.[210] The ability of an individual to develop successfully (and symmetrically) in the face of variable environmental pressures is therefore a proposed indicator of genetic quality, and asymmetric features would be the signature of genetic or developmental problems.[211] Accordingly, a large number of studies in different human societies as well as in macaque monkeys have demonstrated that in a choice test females will prefer males with symmetrical faces over males with asymmetrical faces.[211,212]

Under the principles originally articulated by Darwin, those features that are used by females as signals for male quality should be subjected to sexual selection and become more prominent from generation to generation. This would explain the larger body size of males in polygamous species, but also the development of extravagant ornaments, such as the tail of the peacock, that eventually become a serious handicap for the male's survival in face of predators.

To explain the paradoxical development and survival of such apparently maladaptive features (in morphology or behavior), it has been argued that, in order to be a reliable ("honest") signal for the receiver (the female), a signal has to be costly for the sender (the male), since otherwise there would be an obvious temptation to bluff or deceive the receiver. In this context, the biologist Zahavi proposed the notion of a "handicap principle," according to which females would only (preferentially) pay attention to signals that are costly and could not be afforded by an individual of inferior quality.[213]

Although this principle is not universally accepted, it helps explain several paradoxical aspects of intersexual interactions. One major challenge is of course to formally assess the objective cost of morphological or behavioral signals and their causal relationship with the male's quality. Based on theoretical considerations, and on experimental studies in an insect species, the rhinoceros beetle (*Trypoxylus dichotomus*), Emlen and collaborators suggested that the evolution of trait exaggeration depends on an increased sensitivity to insulin and insulin-like growth factors (IGF) signaling within the growing structure that would amplify otherwise subtle differences in body size growth and condition of the male.[214] Circulating concentrations of insulin and IGFs are sensitive to nutritional status, as well as stress and infection, so that the insulin/IGF pathway can be considered as a central mechanism integrating physiological conditions during growth and translating them into morphological

features in multicellular animal taxa. These authors showed in this rhinoceros beetle that the experimental downregulation (disruption by a double-stranded RNA) of insulin receptor expression differentially affects the development of various body parts (genitalia, wings, forked horn on the head). Wing size was only reduced by 2% but horn size by 16%, confirming their higher sensitivity to perturbations during development.

It is currently unknown whether such a mechanism based on insulin/IGF or another central intracellular signaling pathway is also implicated in the growth of other exaggerated male sexual signals in other species, but insulin/IGF signaling is an ancient (>500 million years) and conserved physiological pathway that connects rates of cell proliferation with available nutrients. It is thus conceivable that this signaling pathway (or another fundamental intracellular signaling pathway) has been repeatedly selected to control the differential growth of signaling structures and make them reliable signals of male quality.

At the physiological level, the preference for specific male characteristics seems to be controlled by the endocrine condition of the female. In humans, it has been demonstrated that women in their fertile phase of the menstrual cycle (high circulating estrogens) demonstrate stronger preferences for masculine men (supposedly carrying "better" genes) than in the nonfertile phases of the cycle (see Ref. 215 for a recent discussion of the status of this controversial research). Preference for more masculine faces around ovulation is associated with an increased interest in extra-pair mating (sexual fantasies about other men, increased receptivity to men's courtship invitations, dressing in more attractive and revealing clothing, etc.) and is interpreted in evolutionary terms as reflecting a search for genetic benefits for offspring.[215]

Indirect Markers of Fertility in Females

Although Darwin developed his theory of sexual selection to account for the evolution of weapons and ornamental secondary sexual characteristics of males that are used to monopolize access to females and determine their mating preference,[5] females also display sexually differentiated characteristics. It is now accepted that the evolution of these features is the result of the competition with other females for breeding opportunities.[216] Male mating preferences vary widely between species, but since males are often able to fertilize multiple females, they do not seen to pay as much attention to the resources that will be provided by the females but rather to their potential fertility and capacity to produce and raise viable offspring. Selection may thus favor the evolution of signals that indicate fecundity. For example, in Barbary macaques (*Macaca sylvanus*), there are discernible differences between mating calls of fertile and infertile females, and calls of fertile females are more likely to attract attention of the males and elicit ejaculation.[217] Similarly, women's voices are rated as more attractive when they are fertile,[218] and one study reported that lap dancers earn significantly more tips from their clients when they are in the follicular (fertile) than in the luteal (infertile) phase. The nature of the stimuli mediating this differential response in men during the fertile period of women (voice, odor, behavior, etc.) was however not identified. Interestingly, this difference was abolished in women taking oral contraceptives that establish a stable endocrine condition across time.[219]

Males thus assess the fertility of their female partner based on indirect markers that reflect the endocrine conditions controlling reproductive physiology, most prominently the circulating concentrations and action of estrogens and progesterone. This idea has been particularly elaborated in the evolutionary psychological literature for the human species. Two secondary sexual characteristics in the human female show this endocrine dependence and have been reported to be major determinants of female attractiveness: (1) the hip-waist ratio, and (2) the enlargement of breasts in females independent of lactation.

Hip Width

In humans, the hip bones are much wider in females than in males, a difference that is not found in other animal species. This sex difference, that is required to facilitate childbirth in a species that has adopted the upright walking position, appears during puberty under the influence of ovarian steroids.[220] The female's broad hips are thus associated with fertility and represent an obvious marker of sexual maturity. They have been considered as an attractive trait of women for thousands of years. Already in the statues of the upper Paleolithic age in Europe (Venus figurines), created some 35,000 years ago, females were represented with precise artistic conventions including an exaggeration of certain parts of the anatomy such as the large hips and breasts. Paleolithic artists had thus already made the link between large hips and fertility, and this morphological feature that is a proxy of the female endocrine condition is still a major feature of female attraction in modern societies.

Breast Size

The human female is the only primate, and even mammalian species, that maintains in a permanent manner fully formed breasts when not pregnant. Females in other species develop full breasts only when there are pregnant. The zoologist Desmond Morris proposed that the shape of the woman's breasts evolved as a frontal, secondary sex characteristic that plays a key role in sexual attraction in relation to the development of frontal copulation.[221]

The development of the breast at puberty and in adulthood reflects the endocrine environment of the subject (mostly estrogens), and it has thus been argued that in ultimate terms, the sexual attractiveness of women's breasts is linked to the fact that they represent a proxy of their fertility.

Furthermore, the breasts' symmetry, like the general symmetry of the human body discussed in the previous section, influences what men and women consider physically attractive in a mate with whom to reproduce. Similar to the general body, breast symmetry is supposed, according to evolutionary psychology, to be a marker of high quality females who developed without interference by disease. Breasts are indeed especially sensitive to developmental interference (genetic and environmental), and breast symmetry thus indicates a woman in good health with a good genetic background, who shall successfully bear more (surviving) children than a woman with asymmetrical breasts.[210] An alternative hypothesis of male attraction to breasts concerns the potential impact of breast stimulation on the neurobiological mechanisms stimulating pair bonding (see following).[222]

Choosing a Healthy Mate

When choosing a potential mate, females often show a preference for a healthy male over a parasitized male. In mice, this choice is achieved through the detection of olfactory signals.[223] There is evidence that the neuropeptide OT plays an important role in the ability of female mice to discriminate between healthy and parasitized mice. For example, female mice can easily distinguish the chemical cues from uninfected males, and males infected with several pathogens, including the nematode, *Heligmosomoides polygyrus*. Female mice show a preference for the odors of the uninfected males, suggesting that those odors are more attractive. Female mice with a disrupted OT gene fail to distinguish healthy and parasitized male odors, or show a preference for healthy males.[224–226] The extent to which OT regulates mate choices based on other features that discriminate better or worse quality mates (e.g., symmetry) is unknown.

Achieving Optimal Outbreeding

It is also advantageous to choose mating partners that minimize inbreeding to avoid its potentially negative influence on the offspring.[227] Males and females are thus faced with the tricky task of selecting as partner subjects who are similar to their father and mother (same species) but sufficiently different to avoid the negative impact associated with genetic similarity.[35,228] Species recognition is often the result, as described in the first part of this chapter, of early learning processes that have been assembled under the term of *imprinting*. But how is inbreeding avoided?

The work of Konrad Lorenz and others indicated that early experiences have a prominent influence on the mating preference in birds and that "sexual imprinting" determines the species with whom an adult will attempt to mate when adult.[15] Although this notion was confirmed and generalized to many other species, including mammals (see first section of this chapter), it was also shown that a bird could show a preference for its own species in the absence of any experience with any congener except itself. Birds could thus have a predisposition to mate with their own species, but if this is the case, then what is the specific role of imprinting? Bateson hypothesized that imprinting is required for the recognition of close kin, allowing birds to select as mates subjects that are similar but slightly different, and thus striking the optimal balance between inbreeding and outbreeding.[229] Working with Japanese quail (*C. japonica*), he tested this idea in a series of experiments and could indeed demonstrate that (1) male quail will mate with slightly unfamiliar females in preference to females to which they were exposed in early life,[229] and (2) more specifically, quail of both sexes prefer first cousins over third cousins, but also over familiar or unfamiliar siblings or novel unrelated birds[230] (Figure 48.12). The dependent variable in these last experiments was the time spent in front of the different stimuli, but they argued that this is a reliable proxy for mating preferences; males were also observed to court the preferred females in these experiments. Taken together, these experiments strongly suggest that

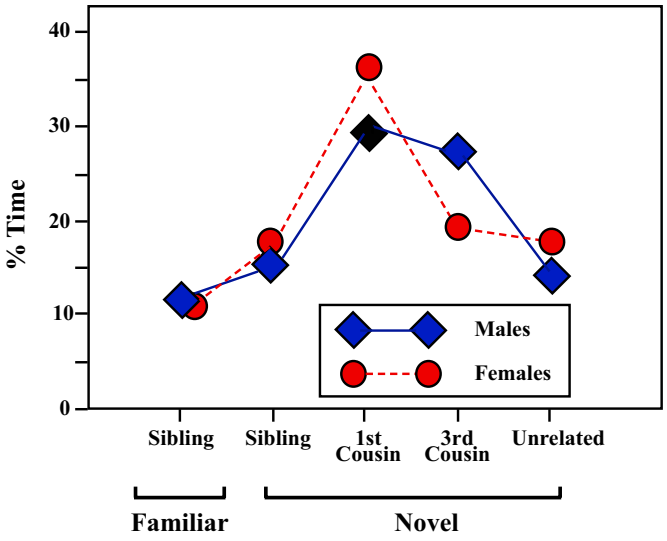

FIGURE 48.12 Mean percentage of time spent by adult male or female Japanese quail near subjects of the opposite sex that were either familiar siblings (birds were raised together) or unfamiliar subjects with varying degrees of relationship (siblings, 1st or 3rd cousins, unrelated). *Source: Redrawn from Ref. 230.*

an interaction between "innate" and learned preferences actually determines the selection of a mate that is similar but not too similar, thus striking the optimal balance between in- and outbreeding.

In mammals, inbreeding avoidance is often controlled by olfactory signals. Remarkably, inbred strains of laboratory mice can discriminate odors of mice that differ genetically only at a single chromosomal region occupied by genes that code for the immune system.[231] This complex of genes is known as the major histocompatibility complex (MHC). These genes regulate important immunological functions and cell surface recognition elements during development,[232] but they are also the source of chemosensory information present in the urine that enables mice to identify one another as individuals.[12,233,234] These odors allow individual recognition but also identification of genetic relatedness, and they are therefore playing a key role in the control of in/outbreeding. Inbred mice were shown to display mating preferences for mice of the opposite MHC type.[233,234] Heterozygosity produced by such matings is suggested to increase the immune system's vigor and range of operation, so that such matings could be of considerable adaptive significance.[232,235]

Individual recognition based on olfactory cues has also been demonstrated in hamsters[236] and in rats.[237,238] Individual preferences then relate to the past sexual interactions: male hamsters prefer the odor of novel females, either mated or unmated[239] while male rats demonstrate a preference for the odor of their previous partner but only until the first ejaculation of a copulatory sequence with a new partner has occurred (see following). After that time, the odor of the novel female will become more attractive.[237] Quite obviously, the formation of stable pair bonds in prairie voles (*M. ochrogaster*) described in the next section of this chapter also requires a capacity for individual recognition, and this recognition is based largely on olfactory signals.[240,241]

MHC-associated mate choice has been suggested in primates and even humans.[242] Although in many primate societies mate choice is constrained by social context, evidence has accumulated indicating that in a variety of sociosexual systems, mating occurs preferentially with subjects possessing a dissimilar/diverse genotype. Primates are supposed to rely less than rodents on olfactory cues in their social and sexual life,[243,244] and these data could be considered as evidence that these cues still play a role in this context. However, the proximate mechanisms mediating this MHC-related mate choice have not been investigated much, and other markers of MHC status (e.g., colors and ornaments, long calls), independent of odors, could be used in the control of mate choice.[242]

In humans, a species that has apparently lost a functional vomeronasal system[245–247] but still retains a functional primary olfactory system, women find the odor of MHC-diverse males more attractive than the odor of less diverse males,[248] suggesting that similar mechanisms still exist in our species. There is also a preference for MHC-dissimilar potential partners.[249] The situation is, however, complex since (1) odor-based studies consistently show a preference for disassortative preferences (preference for different MHC pattern), but (2) in contrast, studies of facial attractiveness indicate a preference for MHC-similar individuals, while (3) analysis of MHC compatibility in actual couples provides conflicting evidence, indicating MHC dissimilarity in some studies but random distribution in others.[249] These results could reflect methodological differences, but could also reflect the fact that the olfactory and visual signals work in a complementary manner to achieve an optimal level of genetic variability in the offspring. The tendency to mate based on visual similarity has been suggested to be influenced by a form of sexual imprinting on the opposite-sex parent during childhood based on studies of visual preference in adopted daughters.[250] More work would definitely be valuable on this fascinating topic.

PAIR BONDING

Introduction

In polygamous species, the decision of who to mate with, followed by the actual mating, is the end of the relationship between the pair. The male immediately seeks another female partner, while the female becomes pregnant and prepares to give birth. However, in monogamous species, a selective pair bond must be developed. Fortunately, in recent years, there has been remarkable progress in understanding the neurobiological and genetic mechanisms leading to the formation and maintenance of the pair bond. Much of this work has been done in the socially monogamous prairie vole. There has also been some work in birds, nonhuman primates, and humans that suggests that at least some aspects of the process leading to pair bonds in voles can be generalized to other species as well. In this section we will detail the current state of understanding of the neurobiology of the pair bond.

The Prairie Vole as a Model Organism

Prairie voles are hamster-sized socially monogamous rodents that evolved in the tall grass prairies of the Midwestern United States (Figure 48.13).[9,251] Prairie voles are highly affiliative and form enduring pair bonds with their mates that typically last a lifetime. Studies in the field have demonstrated that most prairie voles form pair bonds with a single partner, nest together or communally with a single breeding pair, and individuals rarely form a subsequent pair bond if the partner dies.

FIGURE 48.13 Photograph of prairie vole family. Pair-bonded male and female prairie voles share a nest and territory and both parents provide parental care to the offspring. *Source: Photo by Todd H. Ahern.*

In fact, 73% of breakups in prairie vole partnerships are due to death of a partner.[252] Not all prairie voles end up in a monogamous relationship though. Forty-five percent of males do not pair bond and nest with a female, but instead adopt a wandering lifestyle and mate opportunistically with females that they encounter, including partners of other males.

It is thought that social monogamy evolved in prairie voles as an adaptation to habitats with low food prevalence, which results in low population densities. Female prairie voles do not exhibit a regular ovarian cycle like many other rodents, but instead are induced into estrus by the pheromones from a male.[253] Therefore, under low population densities, it is thought that males are unlikely to find a female and be able to stay with her for long enough for her to be stimulated into estrous, and therefore the most adaptive strategy is to bond with a female so that he can immediately impregnate her after she gives birth. However, under higher population densities, wandering males may have an opportunity to produce more offspring, accounting for the variation in mating strategies in natural populations. Male prairie voles share overlapping territories with their partners and display a high degree of selective aggression after they pair bond, and it is thought that this functions to prevent his partner from being mated by a wandering intruder male. However, as we shall see, this may also be an important mechanism to prevent extra-pair copulations with other females as well, which acts to maintain the pair bond. Prairie voles are not strictly genetically monogamous, and extra-pair copulations do occasionally occur.[254]

In contrast to prairie voles, meadow (*Microtus pennsylvanicus*) and montane (*Microtus montanus*) voles are relatively asocial and do not form pair bonds. This species variability in mating strategy among closely related species has provided an opportunity for comparative studies to identify brain mechanisms and genetic elements that may contribute to pair bond formation. Consequently, there is now a significant body of knowledge regarding the neurobiological mechanisms underlying the formation of a pair bond.

Neurochemistry and Neural Circuitry of Pair Bond Formation

OT and Vasopressin

OT and arginine vasopressin (AVP) are evolutionarily related neuropeptides that play an important role in regulating several aspects of social behavior, including pair bond formation. OT and AVP are nine amino acid peptides that differ only by two residues, and originated from a single gene that was duplicated early in vertebrate evolution.[255] Both peptides have important regulatory functions in the periphery. OT stimulates uterine contractions during labor and milk ejection during nursing (see Chapter 13). AVP regulates urine concentration in the kidney and vascular constriction. Both peptides are produced in the paraventricular and supraoptic nuclei of the hypothalamus and projected to the posterior pituitary for peripheral release. However, OT and AVP projections are also found in the brain. We will focus here on those systems that have been implicated in regulating social behavior. OT fibers originating from the magnocellular neurons of the hypothalamus project to several forebrain regions, including the nucleus accumbens (NAcc) and amygdala.[256–259] Many of the AVP projections in the brain originate from extrahypothalamic neurons from the medial amygdala and bed nucleus of the stria terminalis.[260,261] The production of AVP in these neurons is androgen sensitive, and AVP secreted by these neurons regulates male-typical social behaviors.

OT AND PARTNER PREFERENCE FORMATION

The first studies to implicate a role for OT in the regulation of social behavior were focused on the onset of maternal behavior. Pedersen and Prange demonstrated that centrally administered OT resulted in the rapid onset of maternal nurturing behavior in ovariectomized, estrogen-progesterone primed rats.[262] Subsequent studies in sheep by Kendrick and Keverne revealed that OT not only induces the motivation to care for young, but also is involved in formation of the mother–infant bond.[263–265] Infusion of OT centrally to an estrogen-primed ewe causes her to develop a selective bond to a foreign lamb within minutes. OT release in the brain is stimulated by vaginocervical stimulation that occurs during labor and following nipple stimulation during nursing.

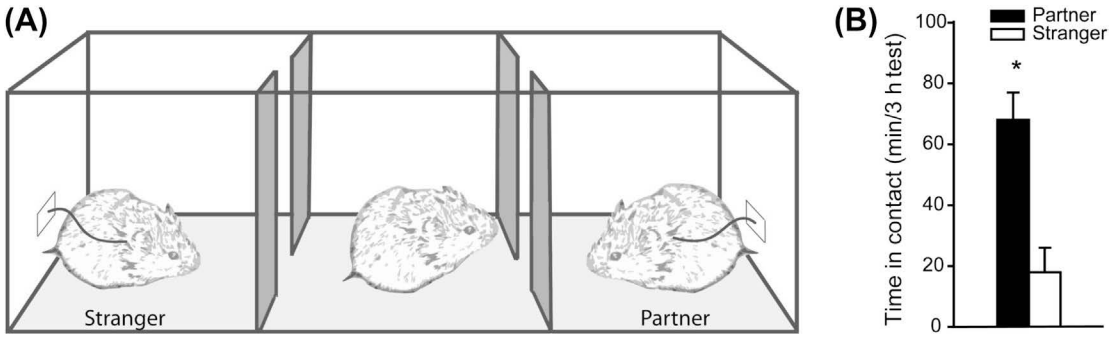

FIGURE 48.14 (A) Illustration of the Partner Preference Test initially developed by Carter and colleagues[266] as a proxy for pair bond formation in prairie voles. Prairie voles are cohabited with an opposite-sex partner for a set amount of time. Mating can be controlled by inducing receptivity in the female with estrogen or preventing receptivity by ovariectomy. Drugs may be infused just prior to or during the cohabitation. The partner preference test takes place in a three-chamber testing arena in which the partner is tethered in one chamber, a novel opposite-sex "stranger" is tethered in the opposite chamber, and the experimental subject is placed in the center and is free to interact with either stimulus animal for 3 h. Time in each cage or time huddling with the partner is quantified. An animal is said to have developed a partner preference if it spends more than twice as much time with the partner than with the stranger. The partner preference test can be easily automated using video-based behavioral analysis software.[270] (B) Typical results following a 24-h cohabitation with mating. *Source: Adapted from Ref. 271.*

OT's role in the formation of the mother–infant bond inspired Sue Carter, Thomas Insel, and colleagues to ask whether OT may also play a role in pair bond formation in prairie voles. Pair bonding in prairie voles is typically assessed using a partner preference test,[266] although mating-induced aggression may also be used as a proxy of pair bonding in males. In a partner preference test, the experimental subject (male or female) is placed in a three-chamber arena in which the partner is tethered to the side of one chamber and a novel stimulus animal of equal stimulus value is tethered to the side of the opposite chamber, with the center chamber empty (Figure 48.14). Females who cohabitated with a male for 6 h without mating typically fail to display a partner preference, defined as spending twice as much time with the partner than the stranger.[266] Cohabitation for longer periods, e.g., for 24 h, without mating can result in a partner preference. However, if the females mate with the male during a 6 h cohabitation, they will display a partner preference.[266] Thus mating facilitates partner preference formation in female prairie voles. However, an intracerebroventricular (ICV) infusion of OT immediately before pairing facilitates the formation of partner preference in female prairie voles after a 6 h cohabitation in the absence of mating. The effect could be blocked by co-infusion of an oxytocin receptor (OTR) antagonist.[267] Furthermore, infusion of an OTR antagonist blocks mating-induced partner preference, suggesting that endogenously released OT is critically involved in partner preference formation in females.[268] A central infusion of OT in males can also facilitate the onset of a partner preference,[269] but one study reported that infusion of an OTR antagonist did not block mating-induced partner preference formation, leading to speculation that endogenous OT does not play a role in partner preference formation in males.[268] However, the role of OT in male pair bonding needs further evaluation as more recent studies using highly selective OTR antagonist suggest that OT is important for partner preference formation in males (A.C. Keebaugh and L.J. Young, unpublished data), although most studies in males have focused on AVP instead (see following).

OT AND SOCIAL INFORMATION PROCESSING

Studies in mice and comparative studies in voles have provided clues as to the precise neurobiological mechanisms by which OT promotes the formation of a partner preference. First, studies in OT knockout mice reveal that both males and females lacking OT display impaired social recognition abilities.[223,272–274] That is, OT knockout mice are incapable of forming olfactory memories to discriminate other mice that they have previously encountered. However, an infusion of OT immediately before, but not following, a social encounter rescues social recognition, suggesting that OT is critically involved in the neural processing of the olfactory signals used to discriminate individuals.[272] More specifically, OT infusion into the medial amygdala rescues social recognition in mice.[272] Since developing a partner preference relies on the ability to learn and recognize the olfactory signature of the mate, it is hypothesized that OT is necessary for partner preference formation because it increases the saliency of the olfactory cues of the partner, facilitating the formation of a memory of the partner.[258] However, the partner preference is more than a memory of the partner, and involves the association of the partner's cues with reward, as we shall see in the following discussion.

OT AND THE REWARD SYSTEM IN PAIR BONDING

Clues to the neural mechanisms by which OT facilitates partner preference formation in female prairie

voles initially came from comparative studies of the distribution of OTR in prairie voles and nonmonogamous meadow and montane voles.[275] Prairie voles have higher densities of OTR binding in the prefrontal cortex and NAcc than do nonmonogamous vole species.[276,277] Furthermore, infusion of OTR antagonists into the prefrontal cortex or NAcc prevents mating-induced partner preferences[278] (Figure 48.15). The prefrontal cortex and NAcc receive rich dopaminergic input from the ventral tegmental area (VTA), and are thus are part of the mesolimbic dopamine reward pathway.[279] As we will see later, dopamine also plays an important role in pair bonding. Given the impact of OT in the neural processing of social signals, and the role of the mesolimbic dopamine pathway in reinforcement learning, it is hypothesized that OT and dopamine interact to form an association memory between the olfactory signature of the partner and the reinforcing aspects of mating.

To characterize OT release in the NAcc in prairie voles, Heather Ross and colleagues at Emory University performed in vivo microdialysis in female prairie voles during cohabitation and mating with a male.[256] OT concentrations in the microdialysates were undetectable under basal conditions when the female was alone, or when a male was placed in the cage, but restricted in a wire cage. However, there was a significant increase in samples with detectable OT in females after they mate with a male. It is likely that the vaginocervical stimulation accompanying the mating stimulated central OT release in a manner similar to that which occurs during parturition.

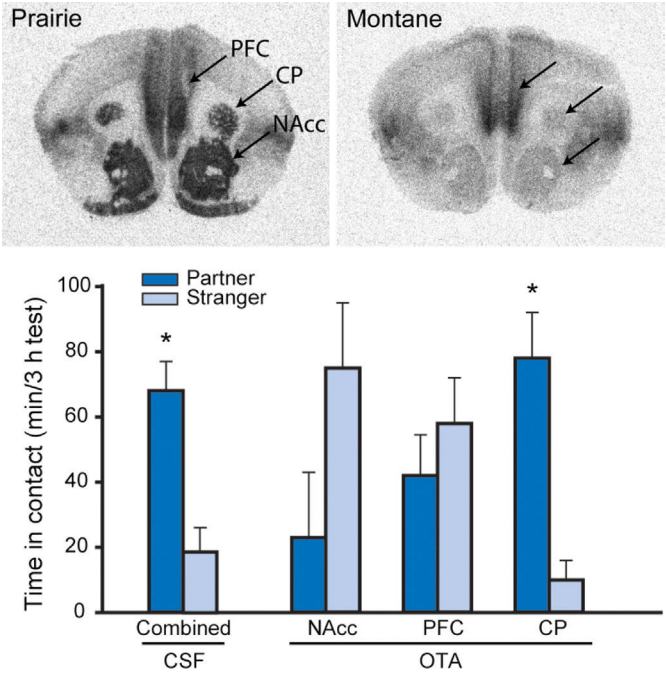

FIGURE 48.15 Receptor autoradiography showing the distribution of oxytocin receptor (OTR) binding in the prairie vole and nonmonogamous montane vole brain. Prairie voles have higher densities of OTR in the nucleus accumbens (NAcc) than do nonmonogamous species. The bar graph illustrates the effect of infusing artificial cerebrospinal fluid (CSF, control) or an OTR antagonist into the NAcc, prefrontal cortex (PFC), or the caudate putamen (CP) on mating-induced partner preference formation in female prairie voles after a 24-h cohabitation with a male. Note the OTR antagonist infused into the NAcc and PFC blocked the partner preference. Asterisks indicate statistically significant differences ($p < 0.05$) in the time spent with partner versus stranger. *Source: Adapted from Refs 277,278.*

There is significant individual variation in the density of OTR binding in the NAcc, and this variation has been associated with variation in alloparental, or spontaneous maternal care.[280–282] There is evidence that individual variation in OTR binding in the NAcc is also relevant to pair bonding. Using viral vectors to increase OTR expression in the NAcc of prairie voles, Ross and colleagues found that artificially increasing OTR expression in the NAcc, thereby making it more sensitive to OT, accelerated partner preference formation in female prairie voles. However, that same study found that increasing OTR expression in the NAcc of meadow voles was not sufficient to confer the capacity to form partner preferences,[257] suggesting that other systems involved in partner preferences are different between these species as well. A study looking at individual variation in mating strategies in prairie vole males in naturalistic enclosures found that males that developed pair bonds had higher densities of OTR binding in the NAcc than did "wandering" males who did not pair bond.[283]

VASOPRESSIN AND PARTNER PREFERENCE IN MALE PRAIRIE VOLES

While OT may play a role in male pair bonding in prairie voles, AVP has received much more attention. In hamsters, AVP stimulates scent marking behavior and territorial aggression.[284–287] It is interesting to consider that AVP's role in scent marking and territorial behavior may be linked to a more ancient role in urine concentration, since urine is commonly used for scent marking.[288] Like OT, AVP also plays an important role in social recognition. Blocking vasopressin V1a receptors (V1aR) inhibits social recognition in rats, and V1aR knockout mice fail to display social recognition.[289–291] The effect of AVP on enhancing social recognition is mediated by V1aR in the lateral septum.[289,291] In male prairie voles, infusion of AVP centrally, via an osmotic minipump, facilitates selective aggression associated with pair bond formation and partner preferences.[292] Furthermore, a selective V1aR antagonist prevents mating-induced partner preference and selective aggression. Central infusion of AVP can induce a partner preference in female prairie voles, but V1aR antagonists fail to prevent mating-induced partner preferences in females.[268] AVP appears to be involved both in the initial formation of the partner preference (acquisition) as well as the expression of

partner preference (recall) but not in the consolidation of the preference. A V1aR antagonist infused centrally just prior to a 24 h cohabitation with a receptive female, or just prior to the partner preference test 72 h after the cohabitation during which time the male was isolated, blocked the display of the partner preference. However, if the V1aR antagonist was given immediately after the cohabitation, 72 h before the partner preference test, the males displayed the usual partner preference.[293]

Comparative analysis of V1aR distributions in monogamous and nonmonogamous voles and behavioral pharmacological studies have revealed two brain regions in which AVP facilitates partner preference formation. Prairie voles have higher densities of V1aR binding in the ventral pallidum, amygdala, and thalamus than meadow or montane voles[294,295] (Figure 48.16). Lim and colleagues infused a V1aR antagonist into each of these regions and found that only blocking the V1aR in the ventral pallidum prevented mating-induced partner preferences in male prairie voles[296] (Figure 48.16).

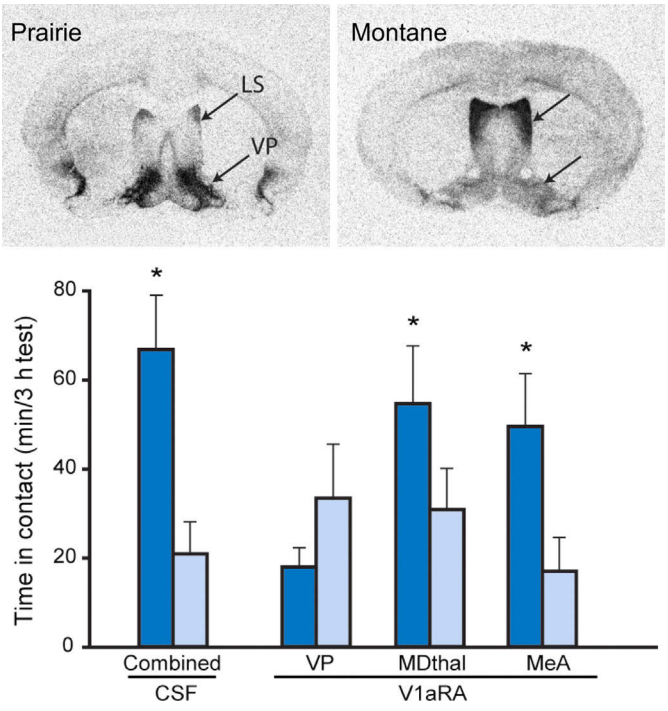

FIGURE 48.16 Receptor autoradiography showing the distribution of vasopressin V1a receptors (V1aR) in the prairie vole and montane vole forebrain. Prairie voles have higher densities of V1aR in the ventral pallidum (VP) than meadow voles. The bar graph illustrates the effect of infusing a V1aR antagonist into the VP, medial amygdala (MeA), and mediodorsal thalamus (MDthal) on mating-induced partner preferences in male prairie voles. Note that the V1aR antagonist only blocked partner preferences when infused into the VP. Other studies have also shown that blocking V1aR in the lateral septum (LS) also blocks partner preference formation. Asterisks indicate statistically significant differences ($p < 0.05$) in the time spent with partner verses stranger. *Source: Adapted from Refs 277,296.*

Furthermore, increasing V1aR density in the ventral pallidum of male prairie voles, using viral vector gene transfer, results in accelerated partner preference formation.[297] Remarkably, when a similar experiment was performed in male meadow voles, to artificially stimulate V1aR densities in the ventral pallidum to the levels found in prairie voles, the meadow vole males displayed a partner preference.[298] This suggests that, at least in voles, the emergence of expression of the V1aR in the ventral pallidum was sufficient to create the capacity to form a pair bond. However, it should be noted that this may not be the mechanism leading to monogamy in other species as density of V1aR binding in the ventral pallidum is not associated with monogamy in several *Peromyscus* species examined.[299]

Like the NAcc, the ventral pallidum is part of the mesolimbic dopamine reward pathway.[279] In fact, the ventral pallidum lies just ventral of the NAcc and is a major output of the NAcc. Interestingly, both the NAcc and ventral pallidum are sites involved in addiction to drugs of abuse, and there are many parallels between addiction and pair bonding as we shall later see.[300] Thus, while different molecules and brain regions regulate partner preference formation in male and female prairie voles, there is a common neural circuit underlying this process.

In addition to the ventral pallidum, V1aR in the lateral septum are involved in partner preference formation in male prairie voles. Infusion of AVP into the lateral septum induced a partner preference in the absence of mating, while infusion of V1aR antagonist into the lateral septum prevents mating induced partner preferences.[301] Interestingly, this study also found that infusion of an OTR antagonist into the lateral septum prevented AVP- and mating-induced partner preference. It is not clear whether this reflects a real role for OTR receptors in this process or promiscuity of the OTR antagonist. As V1aR in the lateral septum are involved in social recognition, which is olfactory based in rodents, the septal V1aR may be critical for processing the olfactory information and formation of a memory of the olfactory signature of the partner.

VASOPRESSIN AND MATE GUARDING IN MALE PRAIRIE VOLES

In addition to the formation of a partner preference, pair bonding in male prairie voles is accompanied by an increase in selective aggression directed to novel males or even females. It is thought that this mating-induced selective aggression serves a mate-guarding function, although this has not been tested. Mating-induced selective aggression is inhibited by a central infusion of V1aR antagonist and is stimulated by an infusion of AVP in the absence of mating.[292] It appears as though V1aR in the anterior hypothalamus mediates

the onset of selective aggression. Gobrogge and colleagues at Florida State University reported that male prairie voles that had been paired with a partner for 2 weeks show an increase in AVP neuron activation in the anterior hypothalamus after a resident intruder test in which the intruder was either a novel male or novel female.[302] They then found, using in vivo microdialysis, that extracellular AVP concentrations were elevated in the anterior hypothalamus of pair bonded males during an aggressive encounter with an unfamiliar female, but decreased during periods of huddling with the partner.[303] Furthermore, infusion of AVP directly into the anterior hypothalamus induced selective aggression in sexually naïve males, while infusion of a V1aR antagonist in the anterior hypothalamus inhibited selective aggression in pair bonded males, demonstrating that V1aR activation in the anterior hypothalamus is necessary and sufficient for the induction of selective aggression.[303] Interestingly, these authors also reported an increase in V1aR density in the anterior hypothalamus of pair bonded males compared to sexually naïve males. They then showed that increasing V1aR expression in the anterior hypothalamus using viral vector–mediated gene transfer increased aggression toward novel females even in sexually naïve males. Thus, it is possible that the increase in V1aR expression in the anterior hypothalamus after pair bonding may serve to decrease the probability that a male will associate, mate, and pair bond with a novel females, although it is clear that pair bonded males do occasionally display extra-pair copulations.

GENETIC MECHANISMS CONTRIBUTING TO DIVERSITY IN PAIR BONDING IN MALES

Since species differences in V1aR receptor distribution in the brain, particularly in the ventral pallidum, are associated with mating strategies in voles, there has been investigation into the genetic mechanisms underlying diversity in V1aR expression. The V1aR is encoded by the *Avpr1a* gene. The species differences in V1aR binding pattern in the brain are likely mediated by differences in *Avpr1a* gene expression since variation in V1aR binding is associated with species differences in *Avpr1a* mRNA.[304] To explore the potential genetic mechanisms underlying this diversity in gene expression, Larry Young and colleagues at Emory University in Atlanta sequenced the *Avpr1a* locus in monogamous prairie and pine voles as well as nonmonogamous montane and meadow voles. While most of the sequences in both coding and noncoding regions were 99% conserved across species, a complex repetitive region, referred to as a microsatellite, in the 5′ flanking region of the gene was strikingly different between the monogamous and nonmonogamous vole species.[305] The microsatellite element is approximately 350 base pairs in the monogamous prairie and pine voles, but only about 50 base pairs in the meadow and montane voles (Figure 48.17). Since the 5′ flanking region of a gene directs gene expression, it was hypothesized that this species difference in microsatellite could be responsible for the species differences in *Avpr1a* expression in the brain. Indeed it was initially hypothesized that the expansion of this microsatellite could be responsible for the evolution of V1aR distribution and therefore of monogamy. However, subsequent analysis of many other *Microtus* species does not support this hypothesis since several nonmonogamous species also have the longer microsatellite.[307] However, there is still evidence, although somewhat contradictory in some cases, that variation in the microsatellite has important implications for behaviors associated with monogamy.[308]

Microsatellites like the one in the vole *Avpr1a* tend to be unstable over evolutionary time scales, which makes them a potential mechanism for generating diversity in gene expression both between and within species. Indeed, there is a significant amount of variability in the *Avpr1a* microsatellite even among laboratory populations of prairie voles.[309] Likewise, there is significant individual variation in V1aR density among wild and laboratory populations of prairie voles.[310] Finally, there is significant individual variation in mating strategies in wild male prairie voles, such that while most males become monogamous resident males with a territory and a single mate, others (about 40%) adopt a "wandering" lifestyle and mate opportunistically with multiple females. Elizabeth Hammock and Larry Young began to explore the relationship between microsatellite length, V1aR distribution, and social behavior by selectively breeding prairie vole pairs with either homozygous long or homozygous short microsatellites. In this laboratory study, Hammock and Young found that, indeed, the length of the microsatellite predicted V1aR density in several brain regions as well as partner preference behavior. For example, male prairie voles homozygous for longer microsatellites have higher densities of V1aR in the lateral septum (which are known to regulate pair bond formation) and were more likely to form a partner preference after cohabitating with a female than males with a short microsatellite[306] (Figure 48.17). Whether the microsatellite affects monogamous behavior in natural settings is more controversial. Alexander Ophir and colleagues examined this in prairie voles in naturalistic enclosures, and while they did find that the microsatellite length affected V1aR densities, they did not find any association with mating tactics or reproductive success.[311] The authors concluded that the precise relationship between microsatellite length, V1aR density, and behavior may be complicated by the fact that microsatellite length is an imperfect metric of the functional elements of the microsatellite that may regulate expression. Other studies in free-living prairie voles, or voles housed in naturalistic enclosures, have also failed to detect a

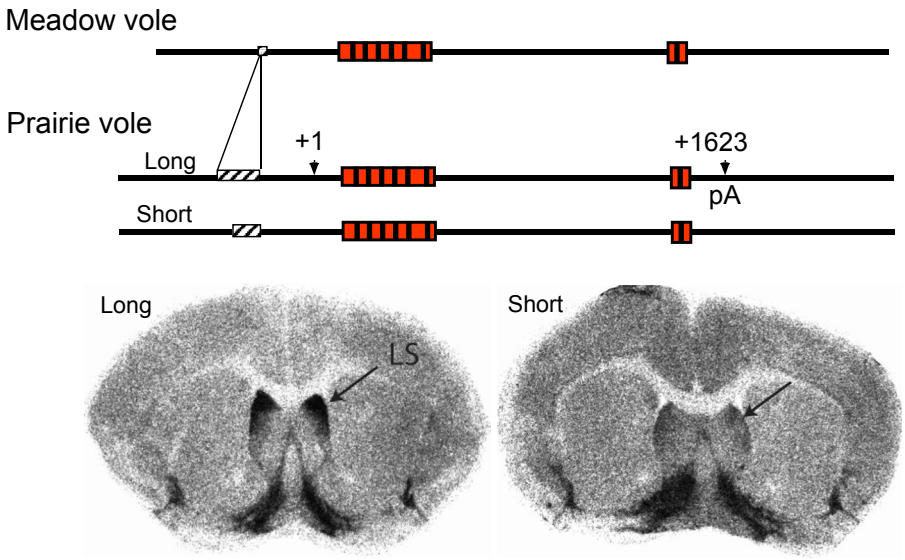

FIGURE 48.17 Schematic of the vasopressin V1a receptor gene (*Avpr1a*) in monogamous prairie voles and nonmonogamous meadow voles. The boxes indicate exons, and the black bars in the exon represent the transmembrane domains of the receptor. The sequences are highly homologous except for a complex repetitive element, referred to as a microsatellite, in the 5′ flanking region of gene (hatched box upstream of the transcription start site, +1). The prairie vole microsatellite is larger than that of the meadow or montane vole. The microsatellite length is polymorphic in prairie voles, and the length has been associated with variation in V1aR binding in the brain as well as behavior in the laboratory, but not in field studies. The brains illustrate the differences in V1aR binding in the lateral septum (LS) between prairie voles with long versus short microsatellites. *Source: Adapted from Refs 305,306.*

strong relationship between mating behavior and the microsatellite.[312,313]

While the precise nature of the impact of variation in the *Avpr1a* microsatellite structure on gene expression and monogamous behavior has yet to be elucidated, more recent studies have further examined these gene-brain-behavior relationships in relation to the V1aR. Ophir and colleagues reported that V1aR density in the posterior cingulate cortex and laterodorsal thalamus, brain regions involved in spatial memory, were correlated with space use and sexual fidelity in animals housed in seminaturalistic enclosures.[314] A recent study used viral vectors expressing shRNA targeting the *Avpr1a* to examine the causal relationship between variation in V1aR density and pair bonding behavior. This approach resulted in a 30% reduction, not an ablation, of V1aR binding in the ventral pallidum, essentially recreating the degree of diversity seen among individuals in wild-caught prairie voles[310] (Figure 48.18). Those males receiving shRNA vector infusions in the ventral pallidum, and thus with reduced V1aR binding in the ventral pallidum, showed a significant impairment in partner preference formation.[315] Finally, Donaldson and Young examined the hypothesis that variation in the *Avpr1a* microsatellite directly impacts V1aR expression in the brain by creating knock-in mice in which 3.5 kb of the mouse *Avpr1a* promoter was replaced with one of three versions of the prairie vole *Avpr1a* promoter. The three versions of the targeting allele differed only in the microsatellite element, which was either a meadow vole microsatellite or a long prairie vole or a short prairie vole microsatellite. Each of these strains displayed significantly different levels of V1aR expression in the brain, confirming that the microsatellite has the potential for diversifying brain V1aR distribution.[316]

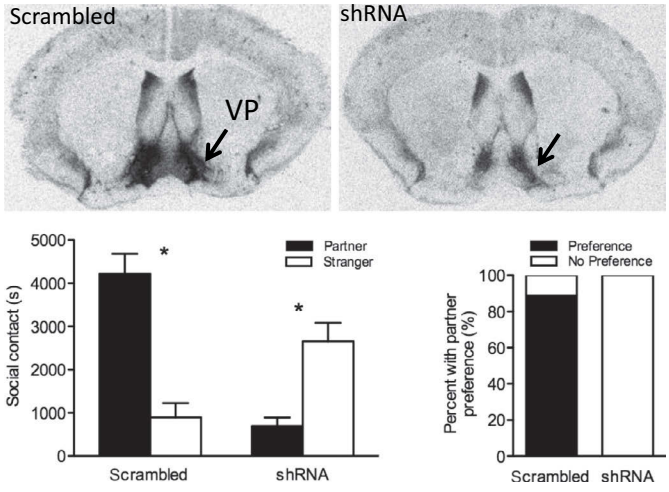

FIGURE 48.18 Viral vector-mediated shRNA knockdown of the prairie vole V1aR in the ventral pallidum (VP) prevents mating-induced partner preference formation in males. Receptor autoradiography reveals an average reduction in V1aR binding in the VP of 30%, consistent with natural variation in V1aR binding, in shRNA-treated males (upper right brain) compared to males injected with a virus expressing a scrambled sequence (upper left brain). This reduction in V1aR binding impaired partner preference formation is illustrated in the bar graphs below. Asterisks indicate statistically significant differences ($p<0.05$) in the time spent with partner versus stranger. These data support the hypothesis that natural variation in *Avpr1a* gene expression contributes to variation in pair bonding behaviors. *Source: Adapted from Ref. 315.*

SEX DIFFERENCES IN THE EVOLUTIONARY HISTORY OF THE NEURAL MECHANISMS UNDERLYING PAIR BONDING

OT and AVP are both involved in the neural processing of social cues required for individual recognition and presumably mate recognition. However, there are some interesting differences in the role of OT and AVP in regulating behavior. It is likely that OT is widely involved in regulating maternal nurturing and maternal bonding in mammals. Thus during the evolution of pair bonding, those neural systems were modified slightly, perhaps through a redistribution of OTR distribution, to facilitate the bonding not only between the mother and the offspring, but also between the female and the male partner who stimulated OT release through mating-induced vaginocervical stimulation. In contrast, AVP in nonmonogamous species is involved in territorial behavior, including scent marking and territorial aggression. Thus it is plausible that these neuroendocrine systems were modified, through an alteration in V1aR distribution, to regulate mate guarding and pair bonding. If this concept is accurate, it would suggest interesting implications for sex differences in the nature of the pair bond between males and females. For example, the female bond with the male may be neurobiologically similar to the nurturing bond of a mother to her infants, while the bond of the male to the female may be the result of the male considering the female as an extension of his territory.[222] While plausible, this is likely an oversimplification as OT may well be critically involved in both male and female pair bonding.

Dopamine and Partner Preference Formation

There is considerable evidence that dopamine plays a critical role in pair bond formation in both male and female prairie voles (for a review, see Young et al.[317] and Young and Wang[277]). Dopamine is involved in reward and reinforcement, and more specifically, reinforcement learning. Wang and colleagues at Emory University first observed that the nonselective dopamine antagonist haloperidol blocked mating-induced partner preferences, while the nonselective dopamine agonist apomorphine induced partner preferences without mating in female prairie voles.[318] Furthermore, a dopamine D2 receptor antagonist inhibited, while a D2 agonist facilitated, partner preference formation when given peripherally or centrally (Figure 48.19). By contrast, a dopamine D1 receptor antagonist failed to inhibit, and D1 antagonist failed to facilitate, partner preference formation.[318] Extracellular dopamine is increased in the NAcc during mating, and site-specific infusion of D2 receptor antagonist into the NAcc, but not into the prefrontal cortex, prevented mating-induced partner preference without affecting mating. Finally, infusion of quinpirole, a selective D2 receptor agonist into the NAcc facilitated partner preference formation in females in the absence of mating.[319] Although both OT and a D2 receptor agonist can stimulate partner preferences when given alone, simultaneous activation of both receptors is necessary for partner preference formation. Liu and Wang demonstrated that infusion of either D2 agonist or OT directly into the NAcc facilitated partner preference formation in the absence of mating. However, an

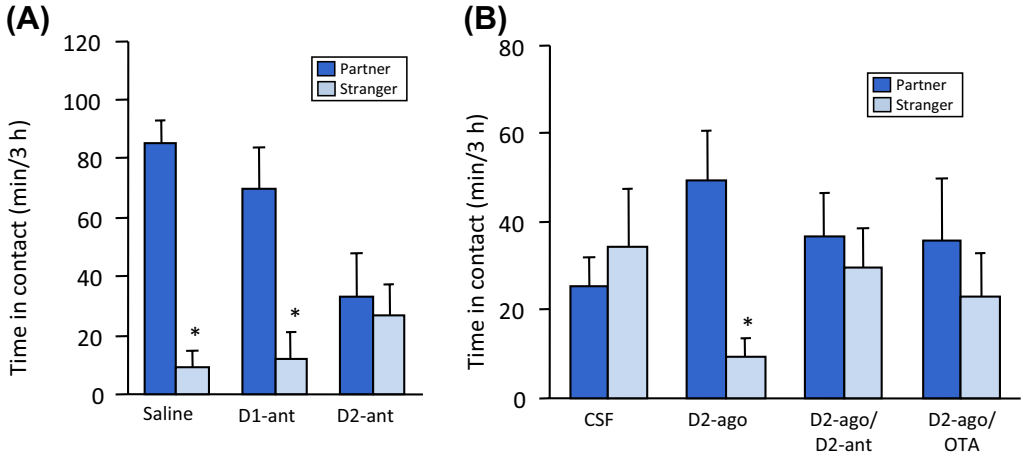

FIGURE 48.19 The role of dopamine in partner preference formation. (A) Female prairie voles were injected intraperitoneally with saline, a dopamine D1 receptor antagonist (D1-ant) or a D2 receptor antagonist (D2-ant) prior to a 24-h cohabitation with mating. The saline and D1-ant groups displayed a robust partner preference, however, the D2 antagonist prevented partner preference formation. It was subsequently shown that D2 antagonist infused into the nucleus accumbens shell blocks partner preference.[319] (B) Female prairie voles that spend 6 h with a partner, without mating, do not form a partner preference under control conditions (cerebrospinal fluid (CSF) infused into the NAcc shell). However, microinjection of a D2 agonist (D2-ago) induces the formation of a pair bond during this 6-h cohabitation, an effect that is blocked by coinfusion of a D2 antagonist (D2-antag). An OT antagonist (OTA) also blocks D2 agonist-induced partner preference, suggesting that concurrent activation of D2R and OTR is necessary for pair bonding. Asterisks indicate statistically significant differences ($p < 0.05$) in the time spent with partner versus stranger. *Source: Adapted from Ref. 277.*

OTR antagonist prevented D2 agonist-induced partner preference, and a D2 antagonist prevented OT-induced partner preference when the drugs were infused in the NAcc of females.[320]

Similar results demonstrating a role of dopamine, including a role for the VTA, have been reported for male prairie voles,[321–323] although an interaction with OT has not been demonstrated. Aragona and colleagues demonstrated that activation of D2 receptors in the rostral shell, but not the caudal shell or core of the NAcc, play a critical role in partner preference formation in male prairie voles.[324] In contrast, D1 receptor activation in the rostral NAcc prevented both mating-induced and D2 agonist-induced partner preferences in males, suggesting that D1 and D2 receptors in the NAcc play opposing roles in pair bond formation.[324] As we shall see later, this opposing role appears to play an important part in preventing partner preference formation in pair bonded males during extra-pair copulations with novel females.

The intracellular mechanisms by which dopamine receptor activation facilitate or inhibit partner preference formation have been investigated by manipulating the downstream signaling, namely cAMP. D1 dopamine receptors are coupled with stimulatory G-proteins, and their activation leads to an increase in intracellular cAMP, which in turn leads to activation of protein kinase A (PKA). Conversely, D2 receptors are coupled to inhibitory G-proteins and consequently lead to a decrease in intracellular cAMP. Aragona and Wang explored the role of changes in intracellular cAMP in the NAcc in male prairie voles by manipulating the production or action of cAMP. Infusion of 3′,5′-phosphorothioate, a PKA antagonist that binds to the cAMP binding site of PKA, into the shell but not the core of the NAcc, facilitated partner preference formation in male prairie voles in the absence of mating in a manner similar to a D2 agonist.[325] These investigators also found that infusing a cAMP agonist (Sp-cyclic adenosine 3′,5′-phosphorothioate), an activator of stimulatory G-proteins (CTX), or a blocker of inhibitory G-proteins, into the NAcc shell blocks mating-induced partner preferences. These data are consistent with the hypothesis that the opposing actions of D1 and D2 receptors on partner preference formation are mediated by their opposite effects on intracellular cAMP signaling.

Opioids and Partner Preference Formation

The opioid system has long been known to mediate the rewarding or positively reinforcing properties of unconditioned stimuli, including food, water, sex, and drugs of abuse. Social stimuli are often rewarding, and the opiate system has been implicated in maternal motivation, infant–mother attachment, and social learning.[326,327] The μ-opioid receptor (muOR) mediates a wide variety of natural rewards, including hedonic "liking" of palatable food.[328] Thus it is not surprising that muOR and the opioid system play a role in pair bond formation as well. Burkett and colleagues found that a peripheral injection of a nonselective opioid receptor antagonist blocked mating-induced partner preference formation, and even induced a stranger preference (or partner aversion) if given repeatedly in female prairie voles.[329] Infusion of the selective muOR antagonist, CTAP, into the dorsal caudate putamen, but not into the NAcc, also prevented mating-induced partner preferences in females (Figure 48.20). This finding was contrary to the initial prediction that NAcc muOR, which mediate the "liking" of food stimuli, are involved in pair bonding. However, the dorsal caudate projects to dopaminergic neurons in the VTA, which in turn project to the NAcc, providing a possible link between the muOR and dopamine systems involved in partner preference formation. An alternative

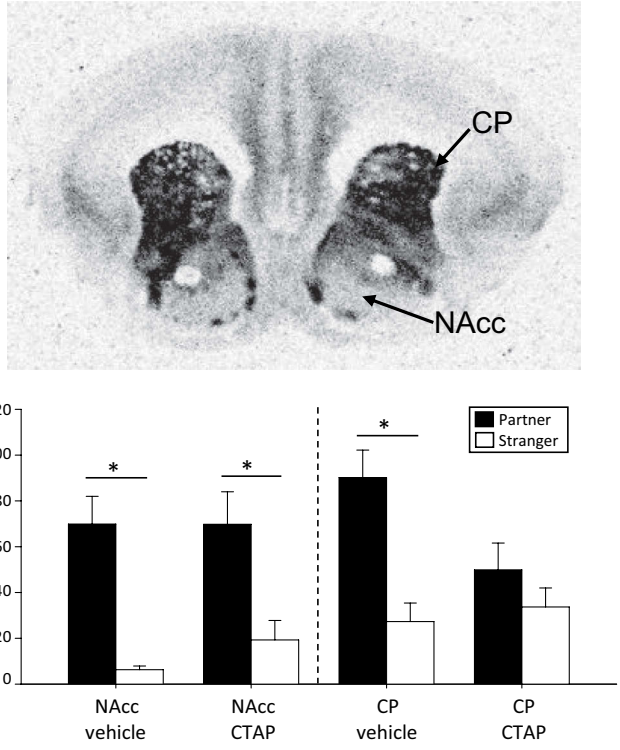

FIGURE 48.20 Mu-opioid receptor (muOR) regulation of partner preference formation. Receptor autoradiography showing muOR ligand binding in the prairie vole striatum. muOR density in the nucleus accumbens shell (NAcc) is moderate, but much lower, than muOR density in the caudate putamen (CP). The bar graph illustrates the effects of infusion of a muOR antagonist (CTAP) or vehicle into the NAcc or CP of female prairie voles prior to 24-h cohabitation with a male partner with mating. Females receiving vehicle to the NAC shell or CP formed pair bonds as normal. muOR antagonist injected into the NAcc shell did not affect pair bonding, while muOR antagonist in the CP prevented the formation of a pair bond. These data show that muOR in the CP is necessary for pair bond formation in female prairie voles. Similar experiments have not been performed in males. Asterisks indicate statistically significant differences ($p < 0.05$) in the time spent with partner versus stranger. *Source: Adapted from Ref. 329.*

explanation is that the dorsal caudate is involved in the shift from goal-directed learning, which involves stimulus-response learning, to habitual learning, which becomes independent of stimulus reward. Pair bonding likely involves a transition from stimulus-response learning, associating the pleasure of mating with the partner, to a more habitual learning where the subject prefers to affiliate with the partner well after mating has ceased.[329]

Partner Preferences and Classical Conditioning

The pharmacological studies outlined herein suggest a neural model of pair bonding involving classical conditioning, utilizing evolutionarily conserved reward learning pathways that have become sensitized to neuropeptides involved in the processing of social stimuli. Nonmonogamous species are also capable of displaying partner preferences under certain circumstances if conspicuous nonsocial cues are provided. James Pfaus and colleagues at Concordia University have shown that both male and female rats form partner preferences similar to that described earlier for prairie voles if repeated mating experiences occur with partners scented with neutral (e.g., almond or lemon), nonsocial scents.[330] For example, if male rats mated over several sessions with a female scented with almond, and are then given a choice between an almond-scented female or a novel female, they will choose the almond-scented female. If the female is not scented with the artificial scent, the males will not show a preference, suggesting that they are incapable of associating the natural social olfactory cues with the reinforcing aspects of mating. Likewise, female rats will display a conditioned partner preference for an artificially scented male if she is allowed to control the frequency of mating through pacing, which is known to be associated with increased dopamine release. Mating-induced conditioning in rats is inhibited by infusion of an opioid antagonist.[330,331]

The ability to associate unconditioned stimuli to conditioned stimuli is an evolutionarily ancient ability. However, some stimuli are more easily paired with sexual reward than others. It is hypothesized that monogamous prairie voles have an exceptional ability to use the complex set of olfactory odors used for individual discrimination as the unconditioned stimulus in sexual conditioning. What is the neural mechanism of this derived capacity? The precise mechanism is not clear, however, it is likely that the localization of OTR and V1aR within the mesolimbic dopamine reward pathway may contribute. As mentioned before, both OTR and AVP are necessary for simple individual recognition in rats and mice. In mice, which do not form pair bonds, OTR in the medial amygdala and V1aR in the lateral septum are essential for individual discrimination.[272,289] Perhaps OTR and V1aR enhance the neural sensitivity to social olfactory cues, allowing for easier detection of individual differences in the mixture of odorant constituents. Endogenous opiates released during mating produces a sense of reward and pleasure, and perhaps stimulates dopamine release. In prairie voles, OTR is concentrated in the NAcc, and concurrent OTR and D2 receptor activation are required for conditioned partner preference formation in females. V1aR is concentrated in both the lateral septum and ventral pallidum, and both V1aR and D2 receptor activation is required. It is hypothesized that the release of OT stimulated by vaginocervical stimulation in female, or AVP in males, results in enhanced transmission of neural coding of olfactory signatures of the partner in the NAcc and ventral pallidum. Simultaneous activation of D2 dopamine receptors in the NAcc, which facilitates reinforcement learning, may strengthen synapses between neurons representing the unique olfactory signature of the partner, and sexual reward.[222,277,332,333] A schematic of this neural model of pair bond formation is illustrated in Figure 48.21.

Stress and Pair Bonding

In addition to the neuropeptide and dopamine systems discussed previously, the stress system modulates pair bond formation in prairie voles. Devries, Carter, and colleagues found that stress has sexually dimorphic effects on partner preference formation.[335] In males, exposure to the stress of a forced swim test, or an injection of corticosterone, just before pairing with a female, facilitated partner preference formation. Furthermore, adrenalectomy inhibits partner preference formation in males. By contrast, swim stress and corticosterone treatment interferes with partner preference formation in female prairie voles.[335,336] Corticosterone interferes with the formation, but not the expression, of the partner preference, since corticosterone given after the cohabitation with the male did not prevent the display of the partner preference. Adrenalectomy facilitates partner preference formation in females even after a 1 h cohabitation with a male.[336] Interestingly, females display a significant decline in plasma corticosterone concentrations after a 60 min or 180 min cohabitation with a male, but not after cohabitation with a female.[336] This decline in corticosterone was not seen in pair bonded females who were exposed to a novel male, suggesting that the decline in corticosterone parallels the formation of the partner preference.

Interestingly, these sexually dimorphic effects of stress axis activation in prairie voles parallel the modulation of the stress axis by OT and AVP. AVP is anxiogenic and enhances stress responses centrally, and stimulates ACTH secretion from the pituitary, and subsequently corticosterone from the adrenals. OT, by contrast, is anxiolytic, and central OT suppresses the HPA axis, inhibiting ACTH and corticosterone secretion.[337] Thus there

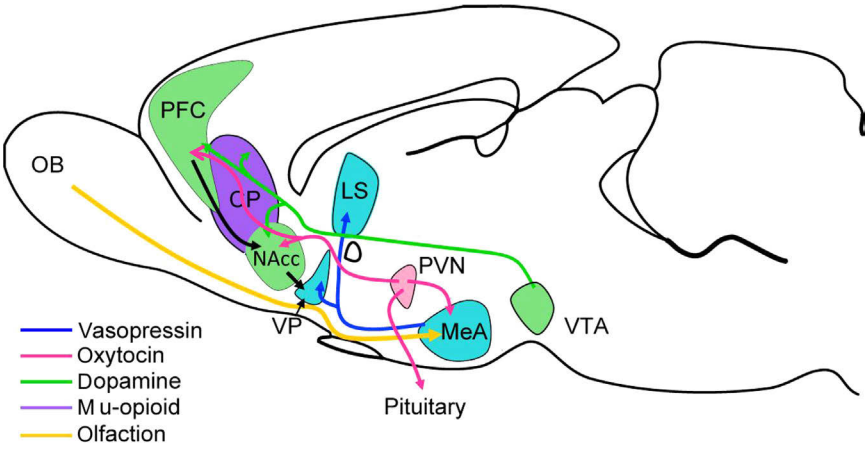

FIGURE 48.21 A schematic of the proposed neural circuitry of pair bond formation in prairie voles. This figure is reproduced in color in the color plate section. Mating stimulates the release of oxytocin from neurons in the paraventricular nucleus of the hypothalamus (PVN) into the nucleus accumbens (NAcc) and prefrontal cortex (PFC) in females, and the release of vasopressin from neurons in the medial amygdala (MeA) into the ventral pallidum (VP) and lateral septum (LS) in males. Mating also stimulates the release of dopamine from neurons in the ventral tegmental area (VTA) into the NAcc and PFC. The MeA receives olfactory input from the partner and conveys the neural encoding of the olfactory signature of the partner to the striatum and LS. Mu-opioid receptors in the CP stimulate the rewarding aspects of mating. OT and AVP facilitate the processing of the olfactory signatures of the mate, and dopamine facilitates an association between the neural encoding of the social cues of the partner and the opioid-based reward and reinforcement. The actions of these neurochemicals in these circuits lead to a conditioned partner preference. *Source: Adapted from Refs 271,334.*

may be an important interaction between the stress axis and the OT and AVP systems in the regulation of pair bond formation. In addition, the effects of OT and AVP on anxiety may also contribute to their modulatory effects on partner preference formation, although this possibility has not been explicitly tested. For example, OT released during mating may decrease anxiety in both males and females, resulting in increased approach behavior.

There is also evidence that the inhibitory effect of corticosterone on partner preference formation may involve an interaction with the dopamine system. RU486, a glucocorticoid receptor antagonist, facilitates partner preference formation in female prairie voles. However, if the females had dopamine receptor antagonist infused into the NAcc, RU486 was ineffective at stimulating partner preference.[338] However, this observation does not preclude an interaction with the OT system, since blocking dopamine receptor inactivation also prevented OT-induced partner preference formation.

In addition to corticosterone, corticotropin-releasing factor (CRF) can also stimulate partner preferences, although it is not known whether endogenous CRF naturally plays an essential role in partner preference formation. CRF produced in the PVN and released into the portal system of the anterior pituitary stimulates the release of ACTH. However, CRF is produced in other brain regions and there are CRF receptors distributed throughout the prairie vole brain that regulate anxiety-related behaviors.[339] ICV infusion of 0.1 or 1.0 ng of CRF facilitated partner preference formation in male prairie voles after a 3 h cohabitation with a female without mating.[340] Higher doses failed to facilitate a social preference. Co-infusion of a nonselective CRF antagonist prevents the CRF-induced partner preference. To further investigate the neural mechanisms by which CRF facilitates partner preferences in males, Lim and colleagues demonstrated that CRF acts in the NAcc to facilitate partner preferences. Infusion of CRF directly into the NAcc, but not into the caudate putamen, facilitated partner preferences in males. This effect was blocked by either a CRF type 1 (CRF-R1) or a CRF type 2 (CRF-R2) receptor antagonist.[341] Immunocytochemistry has revealed that CRF-containing fibers are present in the NAcc.[342] However, there have been no published experiments determining whether infusion of a CRF antagonist prevents mating-induced partner preferences, which is needed to determine whether CRF receptor activation participates in natural pair bond formation.

Neural Mechanisms of Pair Bond Maintenance

Thus far we have discussed the neural mechanisms involved in the formation of a pair bond, many of which involve systems involving positive affective states (e.g., reward). However, recent studies have revealed additional mechanisms that serve to maintain the pair bond that may be acting through modulating negative affective states.[343,344] That is, while activation of the mesolimbic dopamine reward system is involved in the initial establishment of the partner preference, CRF, dopamine D1, and k-opioid receptors all appear to maintain the pair bond by stimulating aversive states when the pair bond is threatened or when the pair is separated.

Dopamine and Selective Aggression

A pair bond in male prairie voles is associated with both a social preference for the partner and an increase in selective aggression toward novel individuals. This aggression toward novel animals, particularly toward females, likely served to prevent subsequent pair bond formation after the initial pair bond is formed. There is evidence that a reorganization of the NAcc dopamine system plays an important role in pair bond maintenance. As mentioned before, D2 signaling facilitates, while D1 signaling inhibits, partner preference formation. Male prairie voles that have mated and been paired with a female partner for 2 weeks display a significant increase in D1-like receptor density in the shell and core of the NAcc, but not in the caudate putamen.[324] This increase was not apparent after only 24 h of mating and cohabitation. Males that mated and cohabitated with a female for 2 weeks display high levels of aggressive behavior toward unfamiliar females. Infusion of a D1 receptor antagonist into the NAcc abolished this selective aggression and increased affiliative behavior toward novel females. Thus, in addition to antagonizing the development of a partner preference, D1 receptors in pair bonded animals facilitate aggression toward novel females, thereby preventing the possibility of pair bonding with additional females. This upward shift in D1:D2 ratio ensures that dopamine release in pair bonded animals leads to greater D1 activation than occurs in non-bonded males during their initial sexual encounter with their partner. But if this D1 receptor-mediated increase in selective aggression prevents extra-pair copulations in pair bonded males, what explains the incidence of infidelity in male prairie voles in natural populations? The authors of this study proposed an interesting possibility. While as a group pair bonded males had a significant increase in D1 receptor binding, 28% of the males in the study did not show a robust reorganization of D1 receptors after pair bonding. Perhaps males that fail to upregulate their D1 receptors are more likely to engage in extra-pair copulations. This possibility remains to be tested.

Kappa-Opioid Receptors and Selective Aggression

The opioid system has also been implicated in the maintenance of the pair bond through the regulation of selective aggression in male prairie voles. Dopamine D1 receptors are expressed on dynorphin-producing neurons. Dynorphin is the endogenous ligand for kappa-opioid receptors (KOR). In contrast, dopamine D2-like receptors are expressed on neurons producing enkephalin, the endogenous ligand for muOR. While enkephalin and muOR are involved in motivation and positive hedonics, dynorphin and KOR are involved in regulating aversion and negative affect. Because of this interaction between KOR and D1 receptor systems, Resendez and colleagues explored the possibility that KOR may contribute to the maintenance of the pair bond in a manner similar to D1 receptors. These investigators found that a peripherally administered KOR antagonist, but not a muOR antagonist, blocked mating-induced selective aggression in males.[345] KOR antagonist was then infused into the NAcc shell, core, and ventral pallidum, all regions showing KOR binding, in both male and female prairie voles. KOR antagonist infused into the NAcc shell, but not the core or ventral pallidum, significantly decreased resident intruder aggression in pair bonded males and females. Thus KOR in the NAcc serves to maintain the pair bond by inducing aggression toward novel individuals of both sexes.

CRF and Separation-Induced Negative Affect

The D1 receptor and KOR systems directly maintain the pair bond by inducing avoidance or aggression to novel individuals, even potential mates. There is also evidence that the CRF system maintains the pair bond by inducing a negative affect upon separation from the partner. Bosch and colleagues investigated the behavioral and physiological impact of losing a partner in male prairie voles. Male prairie voles that were paired with a female for 5 days and then separated from their partner for 4 days displayed a robust increase in passive coping strategy in the forced swim test and tail suspension test, a response indicative of depressive-like behavior, compared to males that remained with their partner.[344] That is, the males spent significantly more time immobile when placed in a beaker of water or when suspended by their tail (Figure 48.22). Interestingly, males that were housed with a sibling for 5 days, and then isolated for 4 days, showed no increase in depressive-like behavior. Furthermore, males separated from their partner, but not from their sibling, displayed a significant increase in corticosterone and adrenal weights. This suggests that important changes occur in the brain mechanisms affecting the stress axis during the pair bonding process that evoke a significant stress response when separated from their partner, which does not occur in nonpair bonded animals experiencing social isolation.

Examination of changes in CRF expression, as a function of pair bonding and separation from the partner, revealed that CRF expression in the bed nucleus of the stria terminalis increases significantly upon mating and cohabitation (and presumably pair bonding) in males, but there was no further increase in synthesis upon separation from the partner. Suspecting that mating and pair bonding increases the set point of CRF expression to essentially increase CRF tone and maximize release upon separation, Bosch and colleagues tested the hypothesis that central CRF release following separation from the partner induces the negative affect and passive coping behavior. When

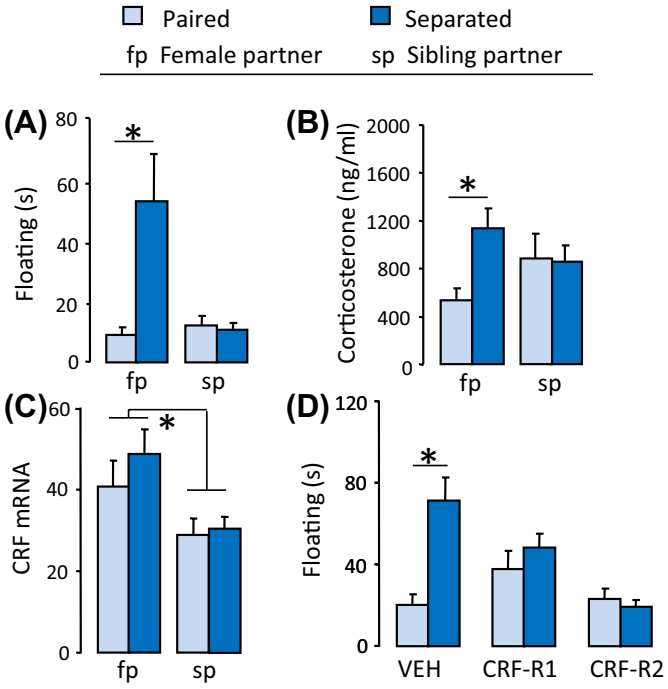

FIGURE 48.22 Loss of a partner leads to an increase in passive coping strategy in male prairie voles. (A) Following 4 days of separation from the bonded partner, males display more time floating (e.g., not actively swimming to escape) in the forced swim test than males who remained paired with their partner, or who were not pair bonded, cohabitated with a same sex sibling, and then separated from their sibling. fp = female partner, sp = sibling partner (B) Males also display an increased plasma concentration of corticosterone when separated from their bonded partner. (C) CRF mRNA expression in the bed nucleus of the stria terminalis is elevated in pair bonded animals compared to nonbonded males. (D) ICV infusion of either a CRF-R1 or CRF-R2 receptor antagonist during the separation period blocks the passive coping strategy in the forced swim test. *Source: Adapted from Ref. 344.*

a nonselective CRF antagonist, a selective CRF-R1 antagonist, or a CRF-R2 antagonist was infused ICV continually via an osmotic minipump beginning on the day of separation, no increase in immobility was observed in either the forced swim test or the tail-suspension test (Figure 48.22). The CRF antagonists did not disrupt the display of a partner preference. Interestingly, CRF-R2 receptors are nearly 100% co-localized with OT neurons in the PVN and are localized to OT immunoreactive fibers in the NAcc, thus there may be an interaction of CRF and OT release in the onset of social loss-mediated depressive-like behavior.[346]

Bosch and colleagues speculate that the CRF system becomes potentiated during pair bond formation, and CRF release during separations from the partner results in a negative affect, which can be alleviated only by reunion with the partner. However, in the case where reunion with the partner is impossible, a maladaptive depression-like behavior develops such that the animal reacts passively in difficult situations. Indeed, in the vast majority of cases where a prairie vole loses their partner in nature, the surviving individual does not take a new partner.

Parallels between Pair Bonding and Addiction

There are several notable parallels between the neural mechanisms of drug addiction and pair bonding, particularly with regard to dopamine, opioid, and CRF systems (for a review, see Ref. 300). Drugs of abuse lead to an increase in dopamine release in the NAcc, as does mating. Chronic cocaine use, which leads to addiction, results in an increase in D1 and a decrease in D2 dopamine receptor signaling in the NAcc, and it has been suggested that the disruption in balance between D1 and D2 receptors underlies the behavioral changes seen in addiction. Opioids are involved in every stage of the addiction process, including initiation, maintenance, withdrawal, and relapse. The positive reinforcing effects of drugs are mediated largely by muOR in the NAcc, ventral pallidum, and VTA. In fact, opioid antagonists have some efficacy at treating drug addiction. During withdrawal, there is an upregulation of KOR, and it is thought that KOR activation by dynorphin may contribute to the negative affect of withdrawal and therefore plays a role in addiction maintenance. Finally, CRF plays an important role in withdrawal. CRF release in the brain is potentiated during withdrawal from a variety of drugs of abuse. This CRF release induces a withdrawal-induced anxiety state that can be revered by a CRF antagonist. This negative affect creates drug craving and is a strong motivation to take the drug.

Thus with the exception of OT and AVP, all of the known mediators of pair bonding are also involved in addiction. Indeed, there is an interaction between social bonding and the effects of drugs of abuse. Amphetamine induces a conditioned place preference (analogous to a conditioned partner preference) in sexually naïve male prairie voles but fails to do so in pair bonded males under the same condition.[347] Based on several pharmacological manipulations, the authors concluded that the pair bonding experience diminishes the rewarding properties of amphetamine through its impact on the D1 dopamine receptor. The difference between drug addiction and pair bonding is the subject of the craving. OT and AVP may serve to link the neural coding of the social cues of the partner to the neural mechanisms involved in addiction through its role in facilitating social information processing.[300]

Pair Bonding as a Model of Social Cognition

Partner preference formation is a complex cognitive process that involves social reward and reinforcement, social information processing, and learning and memory.

As such, it has been proposed that partner preference may be a useful paradigm for screening compounds that may enhance many aspects of social cognition in psychiatric disorders such as autism spectrum disorders and schizophrenia.[348,349] The fact that intranasal OT has been shown to enhance social cognition in autistic subjects supports this notion.[350,351]

With this translational approach in mind, Modi and Young tested whether D-cycloserine (DCS), a partial agonist for the N-methyl-D-aspartate receptor (NMDA) glutamate receptor, would facilitate partner preference formation. The authors used partner preference formation as a proxy for socially reinforced learning. DCS is a cognitive enhancer that has been given peripherally to treat phobias and psychiatric conditions. When given intraperitoneally, DCS facilitated partner preference formation in female prairie voles after a 6-h cohabitation in the absence of mating.[349] DCS did not facilitate a partner preference in female meadow voles. To determine the sites of action in the brain, DCS was microinjected into the NAcc, the amygdala, and the caudate putamen. Infusion of DCS in the NAcc and the amygdala, but not the caudate putamen, facilitated partner preference formation.

In another study, the same authors tested the hypothesis that a drug that is known to stimulate OT release would enhance partner preference. Buspirone, a serotonin 1a receptor agonist, induces a significant increase in plasma OT. As predicted, buspirone injected peripherally facilitated the development of a partner preference in females after a 6-h cohabitation in the absence of mating.[348] Finally, α-melanocyte stimulating hormone (α-MSH) has been shown to stimulate central, but not peripheral, OT release in rats via a melanocortin 4 receptor (MC4R) mechanism.[352] Not surprisingly, melanotan II, an MC4R agonist, facilitates partner preferences in female prairie voles after a 6-h cohabitation without mating (Modi and Young, submitted). Furthermore, even if the partners are separated following the treatment and cohabitation, the partner preference is still displayed 1 week after the drug treatment. Modi and Young suggest that drugs like MC4R agonists, or DCS, enhance socially reinforced learning, and thus might be used in conjunction with behavioral therapies that utilize socially reinforced learning, to improve social functioning in autistic subjects.

Intranasal OT is now being investigated in clinical trials for improving social functioning in autism and schizophrenia. Bales and colleagues have devised a technique to administer OT intranasally in voles to model chronic intranasal OT therapy in psychiatric populations. Intranasal OT was delivered in doses equivalent to those used in clinical studies with autistic patients. Treatment started at 21 days of age, the time of weaning, and continued daily for 3 weeks. Intranasal OT enhanced affiliative behavior toward cagemates in male prairie voles when tested acutely immediately after administration.[353] However, when given chronically and tested 2 weeks after the final treatment, intranasal OT resulted in an impairment of partner preference formation in males, but had no impact in females. The authors conclude that this finding suggests that chronic intranasal OT treatment in humans should proceed with caution as it could have unintended negative effects on social function, but see Ref. 354 for alternative interpretations.

Thus in addition to providing insights into the neural mechanisms of pair bonding and social attachment, partner preferences in prairie voles appear to have important translational potential relevant to disorders characterized by social impairment. Indeed, the prairie vole may prove useful for identifying drugs to improve social functioning in several psychiatric disorders, ranging from autism to bereavement-induced depression.[333,344,355,356]

Effects of Early-Life Experiences on Adult Pair Bonding

There is considerable evidence that early life experiences, including parental nurturing, can have an impact on the ability to form partner preferences as adults.[357] Bales, Carter, and colleagues found that variation in animal husbandry can affect partner preference formation in prairie voles. They compared pups that were transferred during normal cage change in cups or with a gloved hand. Females transferred in cups were less likely to form partner preferences later in life than females handled by a gloved hand.[358] This effect was not seen in males. Although not tested, the authors speculated that the different transfer techniques resulted in differences in parental stimulation.

Variation in family dynamics can also affect later-life pair bonding behaviors. In nature, prairie voles can be reared by a single mother, a breeding pair, or in a communal nest.[9] To investigate the impact of these different types or rearing environments, Ahern and Young created breeding pairs of prairie voles and removed the male in half of pairs just prior to birth of the pups.[359] Daily observations revealed that pups raised by a single mother were exposed in the nest (i.e., no adult was present) more, and received significantly less licking and grooming, than biparentally reared pups. Males and females were then paired with an opposite-sex partner and tested for partner preference after 24 h, 48 h, and 1 week. Biparentally reared males and females displayed partner preferences at all three time points. In contrast, single mother–reared males and females failed to show a significant partner preference after 24 or 48 h, but did after 1 week. No differences between the groups were detected in OTR or V1aR binding, however, the single mother–reared pups had an increased number of OT neurons in the PVN.[359]

Though counter intuitive, this alteration in OT neuron number may reflect some dysregulation in the OT system during development in the single mother–reared pups. Single mother–reared males and females also showed less licking and grooming behavior to their own pups, suggesting a transgenerational impact of early life social experience.[360]

A potential mechanism by which early experience can be functionally translated into altered adult pair bonding behavior is through OT release. There is evidence that parental nurturing and contact stimulates OT release in rodent pups.[361] In male prairie voles, peripheral injections of OT within 24 h of birth leads to increased partner preference formation when the subjects reach adulthood.[362] In females, there appears to be an inverted U-shaped response in which a low dose treatment on the first day of life facilitates partner preferences later in life, a middle dose had no effect, and the highest dose actually led to the development of stranger preferences in adulthood.[363] Furthermore, neonatal OT injections lead to sexually dimorphic alterations in V1aR expression in adulthood,[364] which could explain some of the sexually dimorphic effects of neonatal OT on adult social behaviors.

Pair Bonding in Birds

Despite the fact that 90% of birds form pair bonds and are socially monogamous, and that birds have been widely used to investigate the neural mechanisms of song and communication, there are remarkably few studies investigating the neural mechanisms of pair bond formation in avian species. However, there is some evidence that the avian homolog of OT, mesotocin, and OT-like receptors (VT3) play a role in pair bond formation in zebra finches.

In an initial study, Goodson and colleagues reported that central infusions of mesotocin, vasotocin, and their antagonists did not influence partner preference formation in male or female zebra finches.[365] However, the authors cautioned that the paradigm used may not be ideal for demonstrating that the mesotocin or vasotocin systems are not involved in pair bonding. In a subsequent study involving a seminatural mixed sex setting, an ICV infusion of vasotocin antagonist had no effect on any measure of partner preference formation in male zebra finches.[366] Another group used a testing procedure to allow mate competition and a choice of mates over a 3-day period to test whether an OTR antagonist would alter pair bonding. In this study, peripheral injections of an OTR antagonist disrupted partner preference formation in both male and female zebra finches.[367] Specifically, after 2 days of treatment and three pairing sessions, significantly more OTR antagonist-treated males and females remained unpaired compared to saline-treated controls. However, it is not clear from this study whether the behavioral effects were mediated by central or peripheral mesotocin receptors. Klatt and Goodson followed this study with an experiment where male and female zebra finches were cannulated and given ICV infusions of an OTR antagonist in a colony setting.[368] In contrast to their earlier finding, central OTR antagonist infusion prevented pair bonding in females in this more complex setting, but a decrease in males was not observed. The OTR antagonist used in this study shows a binding distribution, using receptor autoradiography, which is identical to the VT3 receptor mRNA, which is most similar to the OTR of mammals. Thus it appears that mesotocin acting on the VT3 plays a role in partner preference formation in female zebra finches. A role for vasotocin in pair bonding has yet to be demonstrated, and in fact, the evidence does not support a specific role for VT in pair bonding.

There is also evidence that the sex steroid progesterone plays a role in pair bond formation in female zebra finches. Progesterone facilitated pair bonding behaviors in females who had previously never been paired, however, there was no impact on sexual behaviors or behaviors related to parenting.[369] In a second study, male and female zebra finches were allowed to pair bond for 2 weeks, and progesterone was administered on the third week. This study found no impact of progesterone on pair bonding behaviors or extra-pair behaviors. Thus progesterone facilitates pair bonding but does not alter behaviors associated with pair bonding once the bond is formed. It would be informative to determine whether a progesterone antagonist would disrupt an established pair bond. Interestingly, estrogens and androgens do not appear to play a significant role in pair bond formation in zebra finches. When male and female finches were treated with both an aromatase inhibitor (1,4,6-androstatriene-3,17-dione) and an antiandrogen, there was no impact on pairing behavior or the likelihood that an individual would be chosen as a partner.[370]

Pair Bonding in Nonhuman Primates

There are a few studies examining the neurobiological correlates and neurochemistry of pair bonding behaviors in nonhuman primates. Daily intranasal delivery of OT in black-pencilled marmosets (*Callithrix penicillata*) during a 3-week period in which males and females were paired with opposite sex conspecifics resulted in increased huddling with their partner in both sexes, and daily delivery of an oral OTR antagonist caused decreased proximity to their partner.[371] The OTR antagonist also nearly eliminated food sharing with their partner. This study also included partner preference tests similar to those performed in prairie voles, but in this 24-h test, all animals preferentially affiliated with the

novel subject and OT manipulations did not affect this behavior. Interestingly, common marmosets, which are also monogamous, have high densities of OTR in the NAcc.[372] However, it remains to be determined whether these receptors play a role in pair bonding as they do in prairie voles. Nonmonogamous rhesus macaques do not have OTR in this region (S. Freeman and Larry J. Young, unpublished data).

In cotton-top tamarins (*Saguinus oedipus*), urinary OT, thought to reflect plasma concentrations, are correlated with affiliative behavior toward mates in both males and females. Snowdon, Zeigler, and colleagues examined OT concentrations in urine three times per week over a 3-week period in bonded pairs of tamarins and found that OT concentrations varied as much as 10-fold across individuals. However, there was a close correspondence in OT concentration within pairs, and the values were closely correlated. Furthermore, OT concentrations were correlated with affiliative behavior in the pairs. In females, affiliative duration and frequency explained a significant portion of the variance, while in males sexual behavior was the best predictor of urinary OT concentrations. It should be noted that this is a correlational study, and it is not clear if the high concentrations of urinary OT reflect brain levels, which are responsible for the elevated affiliative behavior, or are a consequence of experiencing affiliative behavior, or possibly a combination of the two. Nevertheless, this study suggests a potential role for OT in regulating affiliative behavior in pair bonded primates.

Another study examined the effect of AVP on pair bond related behaviors in coppery titi monkeys (*Callicebus cupreus*) using intranasal administration. Males that had been paired for at least a year with a female were used as subjects. Thirty minutes after an intranasal administration of AVP, the partner, or a novel female, was consecutively placed in the subject's home cage in a smaller transport cage and their interactions were recorded. An empty transport cage was used as a control. Control males receiving saline contacted the stranger's cage more often than the partner or empty cage, similar to the novel partner preference in marmosets mentioned earlier. However, males receiving the highest dose of AVP contacted the partner's cage significantly more than the stranger's cage or the empty cage.[373] While this study did not examine partner preference formation, it does suggest that exogenous AVP induces a preference for contact with an established partner, paralleling the study in voles showing that AVP is necessary for the expression of the partner preference.[293] However, since a V1aR antagonist was not given, it cannot be concluded that endogenous AVP plays a role in partner-directed behavior. This study also does not differentiate between an increased affiliative response toward the partner and an increased neophobia or stress response. However, neophobia is an unlikely mechanism for the preference since there was a clear increase in partner contact and no decrease in stranger contact.

Bales and colleagues also used 2-deoxy-D-glucose PET and MRI to examine neural correlates of pair bonding in titi monkeys. Glucose uptake is thought to reflect neural activity. When they compared males that were in long-term pair bonds to lone males, they found that pair-bonded males had significantly more glucose uptake in the NAcc, ventral pallidum, medial preoptic area, medial amygdala, and supraoptic nucleus (which synthesizes OT and AVP).[374] The authors then compared glucose uptake in animals before and after pairing with a female, and again saw an increase in glucose uptake in the NAcc and ventral pallidum, but not in any other areas. These results are in agreement with the neural mechanisms of pair bonding in male voles, suggesting that similar mechanisms may be involved in pair bonding in rodents and primates.

Pair Bonding in Humans

There is intriguing evidence that mechanisms parallel to those discovered in prairie voles may be involved in romantic pair bonding and relationship quality in humans,[222] however, much of this work is correlational in nature. Feldman and colleagues examined plasma OT concentrations in 60 couples in the early stages of their relationship, within three months, and 43 nonattached singles.[375] Dyadic interactions and interviews regarding relationship-related thoughts and behavior were recorded in a subset of the couples that remained together six months later. Plasma OT concentrations were significantly higher in the new romantic couples compared to the single individuals, and the OT concentrations remained high after six months. Furthermore, plasma OT concentrations were correlated with the couple's interactive reciprocity, including social focus, positive affect, affectionate touch, and synchronized dyadic states. OT was also correlated with anxieties and worries regarding the partner and the relationship. The findings were similar in men and women. While these results are intriguing, and consistent with the work in voles and primates showing a causative role for OT in regulating pair bonding quality, there are two very important caveats to this and other studies examining plasma OT, particularly when the samples are not extracted prior to the assay.[288] First, there is no real evidence that plasma OT concentrations reflect OT concentrations in the brain. However, there is evidence that the OT neurons that are releasing OT from the pituitary are the same neurons projecting to the forebrain, including the NAcc, so plasma OT could reflect neural OT release, but this remains to be determined.[258,259] Second, there is a serious question as to whether OT concentrations determined in

unextracted plasma by commercial ELISA really reflect OT. In fact, one study compared enzyme-linked immunoassay results from unextracted plasma to radioimmunoassay in extracted and purified plasma and found that the values reported by the enzyme immunoassay were 100-fold higher than the gold standard radioimmunoassay results.[376] It is unclear if the values obtained from unextracted plasma samples reflect OT plus its degradation products or also additional unrelated factors, or even OT bound by carrier proteins that is removed in the extraction process.

One intriguing study examined the impact of intranasal OT on personal space in men in a stable monogamous relationship as well as in single men. Scheel, Hurlemann, and colleagues performed the experiment where after intranasal OT or placebo, the male subjects were approached by an attractive female or male experimenter, and the subjects were asked to indicate the distance at which the approach began to be uncomfortable.[377] The authors initially hypothesized that OT would decrease the personal space between the subjects and the female experimenter. However, they found that while OT had no impact on the optimal distance for single men, OT-treated men in monogamous relationships actually displayed an increase in the distance between themselves and the attractive female, but not the male experimenter. The authors suggest that this increased social distance between OT-treated men in a monogamous relationship and attractive females is a mechanism to promote fidelity. Furthermore, women can induce this fidelity-enhancing effect in their partners by engaging in behaviors that stimulate OT release in men, such as sexual intimacy.

Gene-association studies are also consistent with the hypothesis that the neural mechanisms underlying pair bonding in humans are parallel with those discovered in voles. Like in voles, the human V1aR gene (*AVPR1A*) has microsatellite elements in the 5′ flanking region that are polymorphic. In voles, the length of the *Avpr1a* microsatellite influences pair bonding behavior and V1aR binding in the brain, at least in laboratory studies (see previous discussion). Walum and colleagues examined the relationship between the human *AVPR1A* microsatellite and pair bonding behavior in 552 same-sex twin pairs and their spouses or partners.[378] Each subject was assessed with respect to various indices of the quality of the marital relationship, including a partner bonding scale, which is composed of items that correspond to the behavioral patterns observed when measuring features of pair bonds in nonhuman primates. There was a significant association between one of the microsatellites, referred to as RS3, and the partner bonding scale in men but not in women. One particular allele of the RS3 polymorphism, 334, displayed a particularly robust association, and men homozygous for this 334 allele were twice as likely to report a marital crisis in the past year and were also twice as likely to be living with their partner but not legally married. Interestingly, the partners of men carrying the 334 allele reported more dissatisfaction with the relationship. As with most gene-association studies in humans, the effect size of the relationship between pair bonding behavior and the RS3 microsatellite was modest, and this polymorphism clearly does not determine behavior alone, but the fact that an association was detected suggests that the V1aR may contribute to some aspects of romantic relationships in humans.

Walum and colleagues followed the *AVPR1A* study with an examination of the relationship between polymorphisms in the OTR gene (*OXTR*) and the partner bonding scale and other measures of relationship quality in over 2300 subjects who had been in relationships for at least 5 years.[379] A significant association was found between a single nucleotide polymorphism (SNP) in the *OXTR* and scores on the pair bonding scale in women. A similar association was detected between this SNP and scores on another scale of pair relationship quality referred to as the affection scale, in an independent sample of 1240 subjects. Interestingly, these associations were present only in women, not men. Additionally, men married to women carrying the A-allele at this SNP reported significantly lower scores on measures of relationship quality than men married to women not carrying this allele. The study looked at the same SNP in girls and found that carriers of the A allele had more "social problems" than noncarriers, which may be related with the later-life relationship quality issues.

Assuming that the OT system plays an important role in pair bond formation in women (but also in men possibly), it is intriguing to consider whether selection of pair bond formation in humans has shaped human sexuality. The two most potent releasers of OT in the female brain are vaginocervical stimulation and nipple stimulation, which occur during labor and nursing. In most species, females engage in sex only during the times when they are fertile, but human females can be sexually aroused at any time. This allows for sex (and thus vaginocervical stimulation) to occur more frequently, often in a face-to-face position. This lack of obligate linkage of sexuality with fertility suggests that sex may be playing a role in humans other than simply reproduction. Humans are also the only species in which the breasts have become a secondary sexual characteristic and in which nipple stimulation is a common component of sex. It is intriguing to consider that human sexuality has been shaped by evolution to recapitulate the physiological stimuli of birth and nursing, to stimulate OT release and therefore strengthen the bond between the female and her partner.

Brain imaging has also been used to examine the neural correlates of romantic love in humans, and these results are consistent in many ways with the vole

studies.[380] Bartels and Zeki used fMRI to examine brain activation patterns in male and female subjects when they viewed color photographs of their lover, or friends of the same sex as their lover, and then contrasted the two situations to identify brain regions specifically activated by images of the lover. Areas more strongly activated by pictures of loved ones include the middle insula, mainly on the left, the anterior cingulate cortex, the posterior hippocampus, the head of the caudate nucleus, and the putamen.[381] Several areas also showed a decrease in activation while viewing romantic partners compared to friends, including prefrontal, parietal, and middle temporal cortices, the posterior cingulate gyrus and medial prefrontal cortex and the posterior amygdala. This study was followed by a study contrasting the activation pattern of mothers seeing photographs of their infant and lovers seeing photographs of their lovers. As would be predicted based on the hypothesis that pair bonding evolved through adaptations in neural systems involved in maternal bonding, there was a significant overlap in brain activation and deactivation patterns between these two groups.[382] Areas showing similar patterns of activation include the striatum (putamen, globus pallidus, caudate nucleus), the middle insula, and the dorsal part of the anterior cingulate cortex. The VTA and the substantia nigra were also activated by both types of love. Based on these studies, the authors concluded that a push–pull mechanism of attachment—which on one hand deactivates areas mediating negative emotions, avoidance behavior, and social assessment, and on the other hand triggers mechanisms involved in reward—is involved in both romantic and maternal love. The authors also suggested that there was substantial overlap between brain activation patterns when viewing photos of loved ones and the distribution of OTR and V1aR. A more recent study by the same group examined whether brain activation while viewing one's lover would be different between heterosexual and homosexual individuals. The authors found no differences in brain activation patterns between these groups, suggesting that the neural bases for same-sex and opposite-sex romantic love are similar.[383]

Using a very similar paradigm looking at intense, early stage romantic love, another group found activation of dopamine-rich areas associated with reward and motivation, namely the right VTA and the right posterodorsal body and medial caudate nucleus.[384] This group also examined brain activation in people who reported to be in love but were rejected by the loved one. They noted activation in areas associated with gains and losses, craving, and emotion regulation and included the VTA bilaterally, ventral striatum, medial and lateral orbitofrontal/prefrontal cortex, and cingulate gyrus.[385] The results were similar to those found in people who were happily in love. The authors noted the striking similarity to brain regions involved in cocaine addiction, in line with the previous discussion of the parallels between love and addiction.[300]

CONCLUSION

During the last few decades, thanks to progress in neurobiology, molecular biology, and also in animal behavior analysis, we have developed a reasonably coherent understanding of how vertebrates select their mate. In many species of vertebrates, species recognition appears to be based partly on a learning process that takes place during early life (imprinting) and interacts with a hardwired (innate?) knowledge of species-specific characteristics, which places constraints on learning. However, only a small number of species have been investigated in detail, and it is likely that a large degree of species variation in these processes will ultimately be discovered.

The mechanisms that lead to the selection of a sexual partner of the opposite sex have also received much attention both in animals (sex partner preference) and humans (sexual orientation). It has been clearly demonstrated, in rodents at least, that the perinatal endocrine environment plays a critical role in this choice. In humans, clinical and epidemiological studies suggest that the same mechanisms are still active, but at the same time genetic and potentially epigenetic influences appear to play a significant role. Contrary to beliefs that are widespread in the general population, there is currently little experimental evidence demonstrating that postnatal social experiences control in a significant manner sexual orientation in humans.

Finally, the specific subject that will be selected as mate is determined by a variety of criteria that are thought to optimize the success of reproduction and therefore reflect the quality and reproductive potential of the mate. However, to date, there is very little information on the brain mechanisms by which individuals choose the opposite sex conspecific to mate with. There will likely be large sex differences in these mechanisms, especially in polygamous species where the females must be particularly choosy and males can afford to be much less discriminant.

There has been intensive investigation of the neural mechanisms underlying pair bond formation, particularly in the monogamous prairie voles. These studies suggest that the mechanisms of pair bonding share many commonalities with the neural mechanisms of addiction. Indeed, pair bonds can be thought of as conditioned partner preferences in which the cues of the partner have been associated neurally with the reinforcing nature of mating and interacting. The precise cellular mechanisms by which this occurs remain to be elucidated. Despite the great detail provided by studies in the prairie vole, there

is little information in other species. Studies in birds, nonhuman primates, and humans suggest that at least some aspects of the pair bonding processes in voles can be generalized to other species. However, as monogamy, and thus pair bonding, have evolved independently multiple times, it is very likely that there will be species-specific mechanisms underlying pair bonding.

Future Directions

Great progress has been made in understanding the mechanisms of mate choice and pair bonding, but these studies have also revealed many questions that still have no satisfactory answer at this time. These questions concern to various degrees the three aspects of mate choice and pair bonding that were discussed in this chapter.

Species recognition is likely to be quite variable between species, and future studies should try to systematize information collected so far to determine whether there are any evolutionary trends in how species recognition takes place. In particular, it would be fascinating to determine whether the relative role of nature ("innate" identification of species-specific features) versus nurture (learning by sexual imprinting) has changed during vertebrate evolution or as a function of ecological and physiological adaptation.

The topic of sexual partner preference and sexual orientation in humans has attracted much attention. It has been shown, both in animals and in humans, that genetic and prenatal endocrine factors play a significant role in these processes. Their relative importance remains, however, unclear, and in particular, it would be critical to determine whether genetic factors affect sexual orientation by modifying the action of prenatal steroids (changes in receptors, metabolism, receptor co-regulators) or by a direct action on brain development that is independent of steroid action. The relative importance of genetic differences and of differences in gene expression mediated by epigenetic mechanisms obviously also awaits further investigation.

There is substantial clinical and epidemiological evidence suggesting that prenatal testosterone affects, in a significant manner, adult sexual orientation, but to date few experimental data support the notion that individual differences in androgen exposure during prenatal life could have an effect on adult sexual orientation. Baron-Cohen has initiated a program trying to correlate postnatal phenotypic traits with prenatal androgen concentrations measured in amniotic fluid samples that were collected for independent medical reasons. Significant correlations have to date been identified between prenatal testosterone and morphological,[111] behavioral,[386–388] or neuroanatomical[389] differences. Subjects included in these studies are now reaching puberty, and it can be hoped that in the not-too-distant future, it will be possible to analyze potential correlations with adult sexual orientation.

Another area that clearly demands additional work concerns the potential interaction between prenatal (endocrine, genetic) and postnatal (rearing, relation with parents and peers, first sexual interactions) factors in the control of sexual orientation. The role of prenatal factors has now been clearly identified, but they seem to explain only a limited part of the variance (40–50% at best). There is no clear evidence that postnatal factors alone are playing an important role, but suggestive evidence has been collected indicating that they might be important in association with prenatal endocrine modifications.

Individual features that ultimately determine mate choice have obviously been the subject of strong evolutionary pressures so that they provide honest signals of mate quality and reproductive potential. Much remains to be discovered, however, about the nature of these signals, how they are perceived, and how they evolve(d) to ensure transfer of relevant information between potential mates. Monogamous species would be particularly useful for studying partner choice, since the choice of the mate can have a life-long impact on their reproductive success, and thus should be under strict control. Prairie voles have been particularly useful for identifying the mechanisms of pair bond formation and maintenance, but there have been no studies to assess how they choose a mate when there are multiple potential partners.

Regarding pair bonding, it will be particularly important to explore the neural mechanisms underlying pair bond formation and maintenance in a wide variety of species. This would allow us to understand which components of the physiological regulation of pair bonding are general principles and which are species specific. There is likely to be much species specificity in the detailed mechanisms, particularly across diverse taxa. Since 90% of bird species form pair bonds, it is unfortunate that there have been very few studies investigating pair bonding in birds. Those that have been performed suggest that the avian analog of the OT system may be playing a role.

While it is true that we have learned much about the mechanisms of pair bonding in the past 20 years, there are clearly a lot of things we do not know. For example, precisely how do the chemical players affect neurophysiology in the brain regions involved in pair bonding? Electrophysiological analysis to examine neural communication combined with behavioral pharmacological approaches would provide important insights into how OT, AVP, dopamine, opioids, CRF, and their receptors modulate neural circuitry to create and maintain the pair bond. It is also likely that other, yet unidentified, mechanisms play a significant role in pair bond formation.

Future studies should also investigate the extent to which the neural mechanisms of pair bonding can be

generalized to other forms of social relationships. There certainly is a high degree of similarity in the mechanisms underlying pair bonding and parental care and bonding. But might these also play a role in friendships or other relationships? Indeed, there is significant translational potential exploiting partner preference formation to identify drugs to enhance social learning in psychiatric disorders like autism. This potential for biomedical discovery needs to be further developed in prairie voles and other organisms.

The prairie vole model needs to be exploited to its full potential to identify the roles of genes in pair bond formation using state-of-the-art genomic approaches. Genomic tools are being developed and made available to the prairie vole research community. Bacterial artificial chromosome libraries have been developed and used to isolate many of the genes implicated in the pair bond process.[390,391] A genetic linkage map and a panel of gene polymorphisms in prairie voles have been established.[392] The prairie vole genome sequence has recently been made available on a public database. Germ line transgenesis has been successful in prairie voles, but so far this approach has not been utilized to probe the role of specific genes on pair bonding.[393] Finally, inducible pluripotent stem cells have been developed from prairie vole tissue, a first step in the process of developing homologous recombination, which has been so useful in mouse genetics to create knockouts and conditional gene manipulations.[394] As more investigators utilize these tools, the growth in our understanding of the mechanisms of pair bonding shall continue on its impressive trajectory.

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CHAPTER

49

Male Sexual Behavior

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INTRODUCTION

Although the physical effects of hormones were described by Aristotle as early as the fourth century BC, it was Arnold Berthold who, in 1849, published the results of the first experiment that recognized the behavioral effects of hormones, which—apropos of this chapter—focused largely on the hormonal induction of male behaviors. Berthold castrated roosters and implanted into their body cavity either one of their own testes or one from another rooster. They developed into adults with both the physical and behavioral characteristics of gonadally intact roosters: large comb and wattles, sexual mounting of hens, aggressiveness, and crowing. Before this experiment, it was thought that only neural inputs could affect behavior. However, it took many years before "internal secretions" became recognized as having physiological and behavioral effects.

Frank A. Beach was a major pioneer in more modern times. His PhD dissertation in 1937 reported the effects of cortical lesions on maternal behavior of first-time mother rats. He then expanded his research to include effects of brain lesions and of hormones on male rat sexual behavior and published the book *Hormones and Behavior* in 1948. Numerous current behavioral endocrinologists studied with Beach or one of his students. William C. Young, another pioneer in the field, studied the prenatal effects of androgens on male guinea pigs; later, his ideas were extended to rhesus monkeys, with his colleague Robert Goy, who became director of the Wisconsin Regional Primate Center after Harry Harlow. In 1959, a seminal article was published by Charles H. Phoenix, Robert W. Goy, Arnold A. Gerall, and William C. Young: "Organizing Action of Prenatally Administered Testosterone Propionate on the Tissues Mediating Mating Behavior in the Female Guinea Pig." This article reported that testosterone could organize male-typical behaviors in a female. Previous work had shown that hormones can activate behavior patterns characteristic of a specific sex, but now it was shown that the organizational effects of hormones during development could profoundly impact or even determine the behavior of adults. Daniel S. Lehrman extended the research on organizational effects of hormones to ring doves and also emphasized that behavior can feed back to influence hormones, which in turn influence the brain. Study of the organizational and activational effects of hormones continues to be a thriving research enterprise and has been expanded to include the genetic and epigenetic influences on reproduction in a number of species.

More recent research in the area of male sexual behavior has emphasized the physiological bases of erection and the different stimuli and contexts that can elicit erection. Benjamin Sachs pioneered this area and described noncontact erections (NCE) in male rats (elicited by an inaccessible estrous female or her odor or by drugs), touch-based erections (elicited by retraction of the penile sheath of a restrained male rat), and *in-copula* erections. Different hormonal regimens and time courses maintain these types of erections. Elaine Hull and colleagues extended this research area with the discovery and elucidation of the specific neurotransmitters and brain areas that regulate male sexual behavior. They determined that the prosexual (i.e., sexually excitatory) effects of dopamine (DA), glutamate, nitric oxide (NO), and oxytocin in the medial preoptic area (MPOA) are complemented by facilitative effects of orexin/hypocretin on the mesolimbic DA system and opposed by the inhibitory effects of serotonin (5-HT) in the lateral hypothalamus. An important emphasis from the Hull lab and others was the role

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of motivation in male sexual behavior, referred to as appetitive behavior by some. Parallel studies in birds by Jacques Balthazart and Greg Ball extended these concepts outside of mammals. On the consummatory end of male sexual behavior, Lique Coolen and colleagues discovered an ejaculation-specific circuit in the spinal cord and several brain areas while Kevin McKenna's research probed the physiological mechanisms of erection, including the neural innervation of the penis and the various disorders that can result in erectile dysfunction. In the modern era an entire field of research on erection arose out of the discovery that NO initiates the major pathway to erection. NO activates guanylyl cyclase to produce cGMP, which mediates the vasodilation of the penis. Indeed, sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis) inhibit the enzyme, phosphodiesterase 5 (PDE5), which breaks down cGMP, thereby prolonging the vasodilation. Numerous brain and spinal areas, neurotransmitters, and hormones interact in an extensive, highly integrated network to promote sexual arousal and behavior. This chapter will first dissect and analyze that network by its specific components and then integrate those components into functional circuits.

Sexual behavior in animals, including the courtship that precedes it, is characterized by enormous diversity. This diversity assures that mating will occur with the optimal partner at the most appropriate time and place, in order to pass parental genotypes on to the next generation. Sexual behavior is an expression of reproductive physiology that developed days, months, or years earlier, in complex neuroendocrine interactions. Because this chapter focuses on male reproductive physiology, we will emphasize his behavior; however, the contributions of the female cannot be overemphasized. Mating requires the active participation of two individuals, and when mating is viewed from the female perspective, a different interpretation often emerges. However, a male-oriented perspective is warranted in the present endeavor, as long as one is mindful that neither the male- nor the female-centered perspective provides a full description of the complex reproductive interaction.

In this chapter, we summarize recent contributions to our understanding of how hormones, the central nervous system, and peripheral structures interact to regulate male sexual behavior. We begin by describing copulatory behaviors of the most commonly studied species, including the measures used to study them and the conceptual contexts that provide the rationales for those studies. We next review the innervation and functional mechanisms of penile erection and ejaculation. We then turn to the behavioral and physiological effects of gonadal hormones and systemically or intraventricularly administered drugs. Next we summarize the functions of the brain areas most clearly implicated in the control of male sexual behavior, including effects of lesions and stimulation, local hormonal and pharmacological manipulations, and measures of neural activity. Finally, we describe the functional interactions among the structures that mediate male sexual behavior. We conclude with a set of questions raised by previous research that remain unanswered. This chapter is an updating of Hull et al.,[1] Meisel and Sachs,[2] and Hull et al.[3]; we urge readers to consult those publications for more in-depth coverage of some of the older research.

PATTERNS OF SEXUAL BEHAVIOR OF MALE MAMMALS

Description of Behavioral Elements

We begin with a very brief description of the components of mating behavior in species commonly studied in the laboratory. A more thorough description can be found in Refs 3,4. Male animals use species-specific displays to advertise their fitness and suitability as a partner. In rodents, both partners may emit ultrasonic vocalizations, which are mutually arousing. During copulation, receptive females typically remain immobile while the male approaches, clasps her flanks with his forepaws, and begins a series of shallow pelvic thrusts (see Figure 49.1). The female may assume a rigid lordosis posture, with her back flat or concave and her tail deflected to one side. The male usually has one or both hindfeet on the ground, although male macaques use a double foot-clasp mount, in which their hindfeet grasp the female's hindlegs.[5] The male begins a series of rapid, shallow thrusts, with his penis at least partially erect. If the male detects the female's vagina, he will perform a deeper, intravaginal thrust, followed by a rapid, springing dismount. This behavior pattern is used as the measure of intromission in rats and many other rodents. In some species, the male shows repeated intravaginal thrusts throughout the intromission, whereas in ungulates, the male may ejaculate immediately after vaginal insertion. Increased numbers of intromissions preceding ejaculation can increase the number of sperm in the ejaculate and also facilitate sperm transport in the female. Females of some species require sufficient vaginocervical stimulation to ovulate or to trigger a progestational state (reviewed in Ref. 6).

Most male mammals ejaculate only after multiple intromissions. Ejaculation is characterized behaviorally by a deeper, longer thrust and a slow, relaxed dismount. Among canids (dogs, wolves, foxes) ejaculation begins soon after penile insertion and is followed by swelling of the base of the penis, which results in a prolonged "lock" of the male to the female.[7] Technically, seminal emission refers to the transfer of semen into the proximal urethra, whereas ejaculation refers to the forceful expulsion of

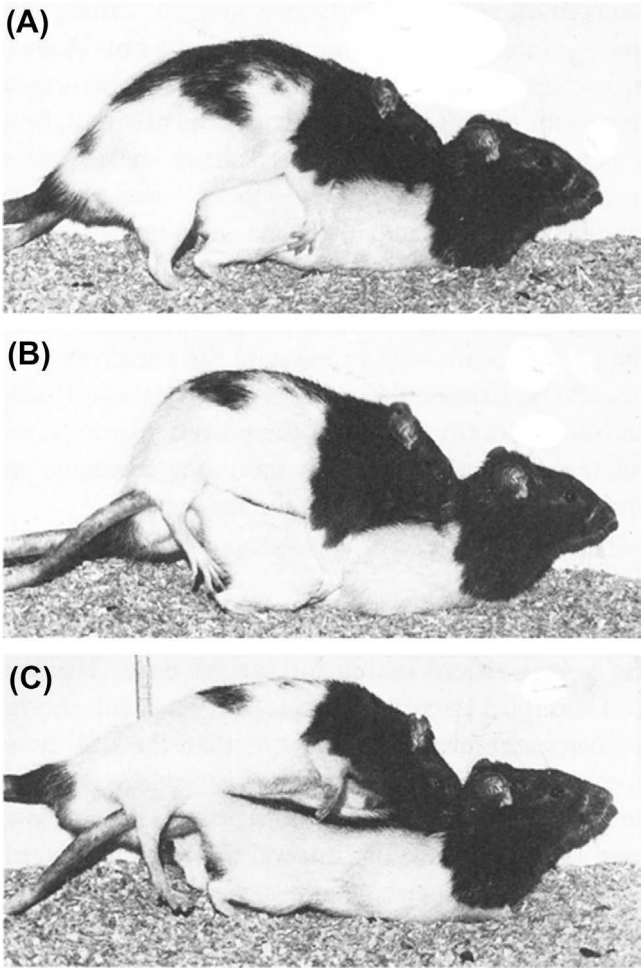

FIGURE 49.1 Three views of copulating rats. Usually, as in A, one is unable to see the genitals. Therefore, without a physiological means of detecting intromission, scoring of copulatory behavior in rats and other small mammals relies upon relatively subtle differences in the patterns of movement to discriminate among mounts, intromissions, and ejaculations. Occasionally the genitals can be seen. In B, insertion is in progress, and the tight clasp suggests that this is an ejaculatory intromission. In C, the engorged glans penis can be seen extending forward just in front of the male's right hindleg. The position of his forelegs indicates that he is dismounting after ejaculation. In all three figures the female is in the characteristic receptive posture of lordosis, with body lowered and rump and head elevated. *Source: We thank Ronald J. Barfield for contributing these photographs.*

the semen from the distal urethra. Rhythmic contractions of skeletal and striated perineal muscles, including the bulbospongiosus, ischiocavernosus, and anal sphincter, usually accompany ejaculation. In human males and females, such muscle contractions are associated with orgasm.

Postejaculatory Behavior and Satiety

After ejaculation, the male typically grooms himself and enters a period of sexual quiescence. The postejaculatory interval may be as short as 30 s in Syrian hamsters or as long as hours to days in other species[8]; in rats it lasts 5–10 min. During the postejaculatory interval, male gerbils footstomp and rats emit ultrasonic (22 kHz) vocalizations. In rats, the failure to copulate during the postejaculatory interval does not result from a failure of erectile function; indeed, *ex-copula* erections may even be enhanced after ejaculation.[9] After achieving seven to eight ejaculations most male rats reach sexual satiety, during which they may not copulate for 1–3 days. Recent data showing a dramatic reduction in androgen receptors in the medial preoptic nucleus and related areas, unrelated to circulating androgen levels, led the authors to suggest that this reduction may underlie the sexual satiety.[10]

Effects of Sexual Experience

Previous sexual experience increases sexual "efficiency." Experienced males copulate with shorter latencies to mount, intromit, and ejaculate and with fewer mounts and intromissions preceding ejaculation. Furthermore, experienced males may be less susceptible to the effects of castration, various brain lesions, and exposure to a novel environment (reviewed in Refs 3,4). In addition, ejaculation elicited more cells that were immunoreactive for Fos, the protein product of the immediate-early gene *c-fos* and considered to be a marker of neuron activation, in the MPOA[11] and in the shell region of the nucleus accumbens (NAc)[12] of sexually experienced male rats, compared to sexually naive males. Similarly, in Japanese quail previous plus acute sexual experience in the presence of a conditioned stimulus (CS) increases Fos immunoreactivity in two brain areas that are important for mating (MPOA and bed nucleus of stria terminalis, BST), compared to exposure to that CS without mating or mating without the CS.[13] Therefore, the threshold for neural excitation is reduced in experienced males, especially in a context associated with mating.

MEASURES OF PENILE FUNCTION

Observations during Copulation

The most direct method to study penile erection is observation during copulatory behavior. This is relatively straightforward in many species, but can be difficult in rodents, because erections are very brief and often obscured from view. Erection, intromission, and ejaculation are often assumed based on the animal's behavior. Direct observations of penile erections can be made using a mirror placed beneath a clear cage. Because genital reflexes are difficult to measure while the male is copulating, paradigms have been developed for monitoring them *ex copula*. However, *in-copula*

and *ex-copula* erections may differ significantly in their physiological, CNS, neurochemical, or hormonal control.[14] To overcome these problems, techniques have been developed to measure physiological responses during copulation, including chronic implantation of electrodes to measure the electrical activity of the striated perineal muscles.[15]

Ex-Copula Measures of Penile Function

Spontaneous or Drug-Induced Erections

Erections can often be observed when a male is alone in his home cage or a neutral arena, in the absence of an apparent sexual stimulus. Therefore, these erections are referred to as spontaneous. The number of spontaneous erections can be increased by administration of various drugs systemically or into specific CNS sites. These erections are referred to as drug-induced erections. These usually involve extension of the engorged glans beyond the sheath and are typically accompanied by genital grooming.

Noncontact Erections

Male rats develop penile erections (noncontact erections; NCE) in response to the presence of an estrous female even when physical contact is prevented; volatile odors from the estrous female are necessary and sufficient for this response.[16,17] Their motor pattern is similar to spontaneous and drug-induced erections and may be the closest model for psychogenic erections in humans. This model is an important contribution, because the response is produced by supraspinal mechanisms and not spinal reflex pathways.

Touch-Based Erections, Anteroflexions, and Seminal Emissions

Reflexive, or touch-based, erections have been elicited by manually stimulating the penis, primarily in the dog.[18] However, penile erection is inhibited by tactile stimulation of the penis in rats.[19] A different method for evoking erection in rats was developed by Hart.[19] A similar technique has been described in mice.[20] With the rat restrained on his back, the penile sheath is retracted, exposing the glans penis. This gives rise to a series of erections, which are seen as tumescence of the glans penis due to engorgement of the corpus spongiosum (see Figure 49.2). Penile anteroflexions ("flips") also occur. These are caused by erection of the corpora cavernosa and contractions of the ischiocavernosus muscles, causing the penis to rise up from its normal posteroflexed position. The *ex-copula* and *in-copula* erections are both based on parasympathetically mediated vasodilation in the penis and contractions of the striated penile muscles (reviewed in Ref. 3). It is thought that the continuing pressure of the sheath around the base of the penis provides the sensory basis.

Urethrogenital Reflex

A model for both erection and orgasm, the urethrogenital (UG) reflex, has been described in anesthetized male and female rats.[22] In urethane-anesthetized, acutely spinalized male rats, the UG reflex consists of clonic contractions of the perineal muscles, rhythmic firing in the cavernous nerve, penile erections, and ejaculation (reviewed in Ref. 3). The somatic muscle bursts are synchronized with bursts in the cavernous nerve, driven by both pelvic (parasympathetic) and hypogastric (sympathetic) nerve bursting, indicating a multisegmental organization. This reflex can be evoked only in animals

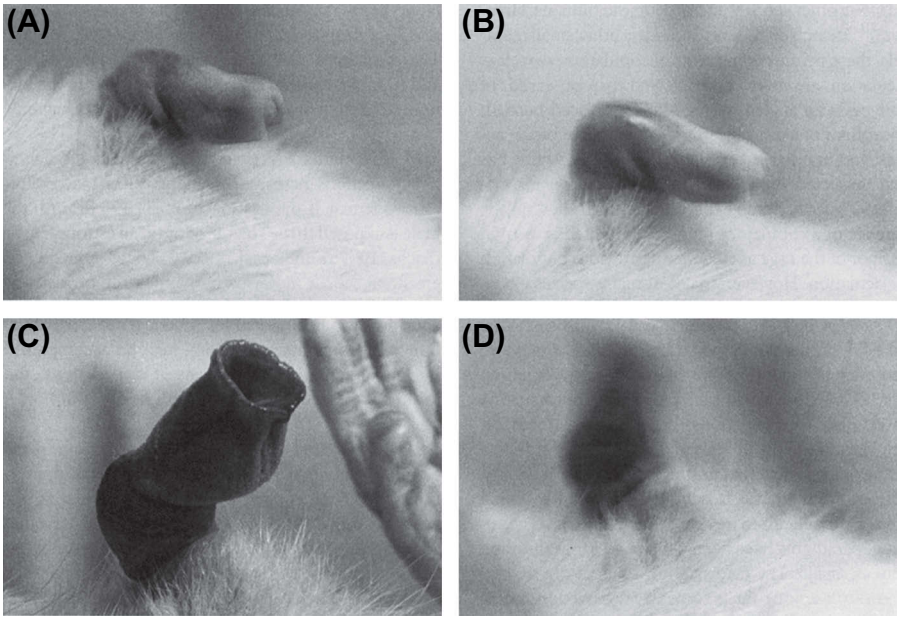

FIGURE 49.2 **Reflexive erections displayed by supine male rats after retraction of the penile sheath.** Several days earlier the suspensory ligaments had been surgically removed, thereby preventing retraction of the penis during the test and permitting better visualization of the distal penile body (perpendicular to the torso in B) and of the entire glans (parallel to the torso in B). In A to C, the glans is directed toward the tail, its normal orientation. In D the glans points rostrally, the orientation necessary to achieve intromission. (A) The quiescent (flaccid) penis. (B) Tumescence and elevation of the penile body without glans erection. (C) Intense erection of the glans ("cup") and of the penile body. (D) Anteroflexion ("flip") of the penis due to a straightening of the penile body. The blurring is due to the speed of the response relative to the shutter speed (1/30 s). *Source: The photographs are from Ref. 21, with permission.*

that have high spinal transections or lesions in certain brain areas.

THE ROLE OF THE PENIS IN MALE SEXUAL BEHAVIOR

Anatomy of the Penis and Mechanics of Erection

The anatomy of mammalian penises varies between a fibroelastic and a highly vascular organ. In ungulates such as sheep and goats, erection occurs primarily by extrusion of the fibroelastic penis by the action of penile muscles. In other animals, including rodents, cats, dogs, monkeys, and humans, erection involves penile enlargement and stiffening, produced by coordinated vascular relaxation and contraction of striated penile muscles. The relative importance of these two components differs across species.

The general arrangement of the penis is common among all mammalian species (see Figure 49.3). The erectile structures consist of the paired corpora cavernosa, which occupy most of the shaft of the penis, and the corpus spongiosum, which surrounds the urethra and expands at the end into the penile bulb and the glans penis (reviewed in Ref. 3). In most species, the two corpora cavernosa are fused into a single vascular

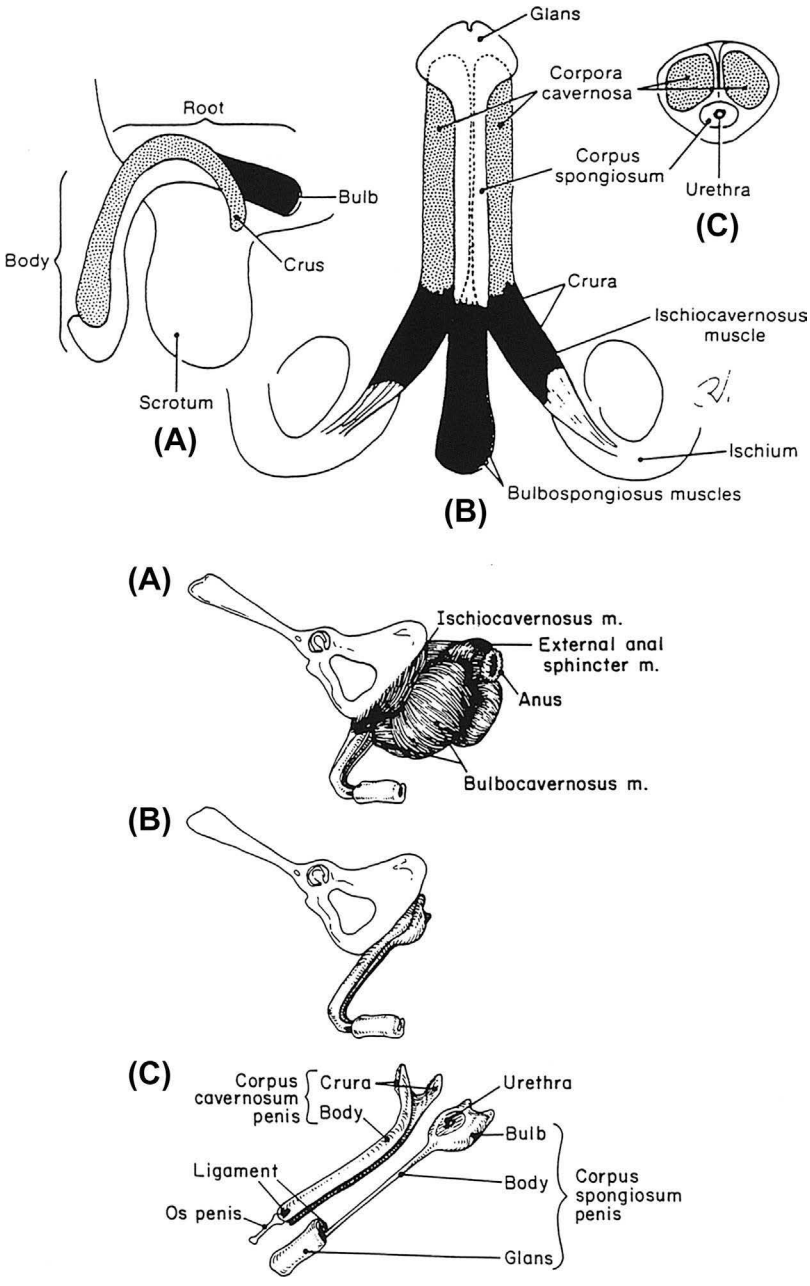

FIGURE 49.3 Some basic structural similarities in penile anatomy for the human and rat. Human (top: (A) lateral view; (B) inferior view with penile body lifted; (C) cross-section) and rat (bottom: (A) lateral view including all muscles; (B) lateral view with muscles removed; (C) "exploded" view). In both species, the penile crura, attached to the ischium, are covered by the ischiocavernosus muscles and are continuous with the body of the penis, composed primarily of the paired corpora cavernosa. The penile bulb, wrapped by the bulbospongiosus (bulbocavernosus) muscles, gives rise to the corpus spongiosum, which terminates in the glans penis. The rat muscle identified as the external anal sphincter is also known as the levator ani or the dorsal bulbospongiosus. *Source: Figures of human penis from Ref. 23 are reprinted by permission of Oxford University Press. Those of the rat penis from Ref. 24 are reprinted by permission of Pergamon Press.*

space, which allows a drug injected anywhere into the corpus cavernosum to reach the entire structure. The corpora cavernosa are composed of vascular sinuses (trabeculae), which receive blood from the helicine arteries, which in turn are fed from the cavernosal artery. The trabeculae are drained by subtunical venules that ultimately join to form the cavernosal veins. The corpora cavernosa are surrounded by a tough capsule, the tunica albuginea, which allows the penis to become hard when the corpora are filled with blood. The proximal ends of the corpora taper into tails called crura, which are anchored onto the ischiopubic ramus and surrounded by the ischiocavernosus muscles. The corpus spongiosum is spongy erectile tissue, which provides a cushion to allow semen to be expelled during ejaculation. Its proximal portion is surrounded by the striated bulbospongiosus muscle. Some animals' penises (but not humans') contain a bone (the os penis), which extends forward to the glans.

The erection and detumescence of the corpora cavernosa and corpus spongiosum are usually coordinated. However, in some conditions they may act independently, because there is some independence of their vascular[25] and neural[26] systems. In humans, erection usually refers only to the penile shaft. However, in rats and other rodents, *ex-copula* erections refer to engorgement of the glans, and changes in the penile body are called anteroflexions or flips. Furthermore, intense erections in rats result in the formation of a cup at the end of the penis, which fits around the female's cervix and directs the flow of semen into her uterus.

Erection is caused by relaxation of smooth muscles in the arteries supplying the erectile tissue and in the trabeculae. The resultant inflow of blood fills the corpora, increasing intracavernous pressure and occluding the venous outflow, trapping blood in the corpora. Contraction of the striated muscles overlying the penile bulb and crura increases penile rigidity.

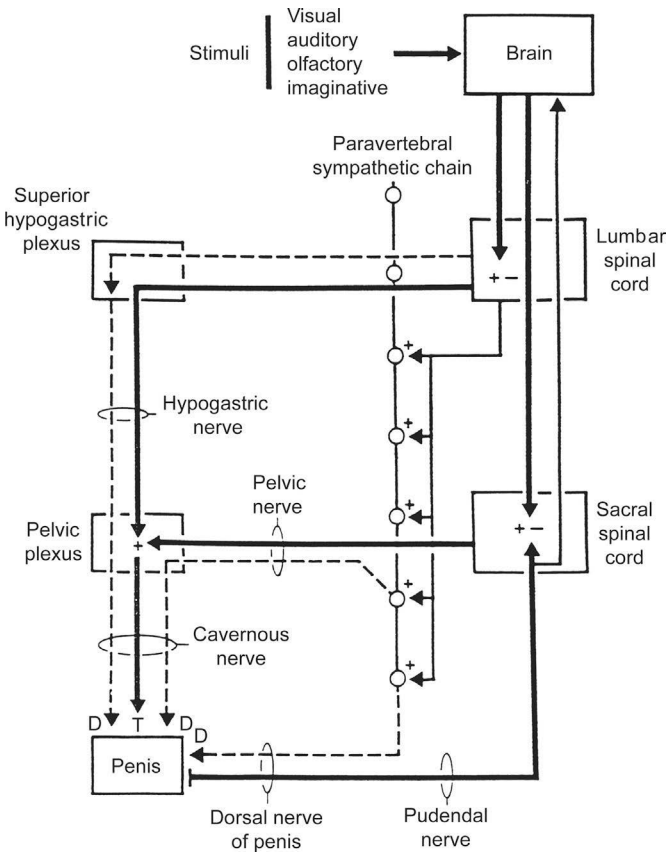

FIGURE 49.4 Schematic diagram of the excitatory (+) and inhibitory (–) innervation of the penis for the regulation of tumescence (T) and detumescence (D). Note that the motor branch of the pudendal nerve is not depicted, nor are the striated muscles to which the pudendal nerve projects. Also, the penis is shown as a single structure, although recent evidence suggests that the innervation of the penile body and glans are not identical. The depicted innervation may better represent that of the body than that of the glans. *Source: Diagram is from Ref. 27 and is reprinted by permission of Harwood Academic Publishers.*

Neural Innervation of the Penis

Penile erection requires coordination of sympathetic, parasympathetic, and somatic input to the penis and perineal structures (see Figure 49.4). The three major pathways include the pelvic nerves (primarily parasympathetic and proerectile), the hypogastric nerves (primarily sympathetic and antierectile), and the pudendal nerves (somatic sensory and motor). The pelvic nerve originates in the lumbosacral spinal cord and travels via the pelvic plexus and cavernous nerve to the corpora and vasculature of the penis. Although it is primarily parasympathetic, it also carries some sympathetic postganglionic fibers, at least in rats.[28] This is the major proerectile pathway in all mammals studied so far.[29,30] The pelvic plexus sends sympathetic and parasympathetic axons to the penis via the cavernous nerve.

The bulbospongiosus and ischiocavernosus striated muscles are innervated by the pudendal nerve, which originates in Onuf's nucleus, in the ventral sacral cord. The pudendal nerve divides into distinct sensory and motor branches near the ischium, both of which also carry sympathetic fibers.[31] The contraction of these muscles does not elicit erection in a flaccid penis, but can dramatically increase penile rigidity of an erect penis.[32]

The third pathway is the sympathetic innervation of the penis, which reaches the penis via the lumbar splanchnic nerves or via the paravertebral sympathetic chain.[33] They synapse on neurons in the hypogastric plexus or in the paravertebral sympathetic chain ganglia. Sympathetic postganglionic axons reach the penis through the hypogastric nerve or the paravertebral sympathetic chain and use norepinephrine (NE) as their neurotransmitter. There are also anti-erectile sympathetic neurons in the

paravertebral sympathetic chain,[34–36] which tend to keep the penis in a flaccid state. Indeed, intracavernous injection of the α-adrenergic antagonists phenoxybenzamine and phentolamine elicits erection in humans.[37]

However, sympathetic neurons may also promote erection.[38] Stimulation of the MPOA elicited penile erection (see Figure 49.5). Bilateral section of the parasympathetic input to the penis blocked the erectile response, confirming that the primary proerectile influence is the parasympathetic innervation. However, section of the paravertebral sympathetic chain or destruction of sympathetic fibers by the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) significantly decreased the erectile response. The investigators suggested that stimulation of the MPOA may have increased vascular tone in other pelvic structures, thereby diverting blood away from those structures and toward the penis. Therefore, the MPOA can coordinate both parasympathetic and sympathetic influences to elicit erection.

Autonomic and Somatic Control of Ejaculation

Ejaculation consists of both emission and expulsion phases. Emission includes parasympathetically controlled secretion of seminal fluids from epithelial cells and accessory sex glands, as well as sympathetic responses that move those fluids to the proximal urethra (reviewed in Ref. 3). The emission pattern includes closure of the bladder neck and contraction of the seminal vesicles, prostate, and ductus deferens.[40] Emission is primarily controlled by sympathetic mechanisms, with some contribution from parasympathetic nerves (see Figure 49.6).

Expulsion of the semen results from rhythmic contractions of smooth muscles of the urethra and striated perineal muscles, primarily the bulbospongiosus muscle. Both sympathetic and somatic outputs contribute. Ejaculation is initiated primarily by sensory receptors of the glans penis (reviewed in Ref. 42). However, ejaculation during nocturnal penile tumescence may not require

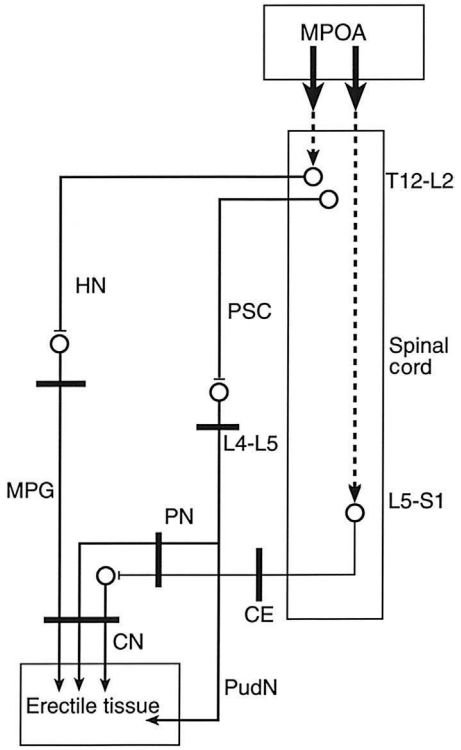

FIGURE 49.5 Diagrammatic representation of peripheral autonomic pathways potentially involved in erectile response elicited by medial preoptic area (MPOA) stimulation. Sites of neural lesions are represented by black bars. No direct projections from MPOA to spinal autonomic nuclei have been reported; dotted lines represent hypothetical pathways. CE, cauda equina; CN, cavernous nerve; HN, hypogastric nerve; L4-L5, 4th and 5th lumbar levels of the paravertebral sympathetic chain; L6-S1, 6th lumbar and 1st sacral level of the spinal cord; MPG, major pelvic ganglion; PN, pelvic nerve; PSC, paravertebral sympathetic chain; PudN, pudendal nerve; T12-L2, 12th thoracic to 2nd lumbar level of the spinal cord. *Source: Figure is from Ref. 39 and is reproduced with permission from the American Physiological Association.*

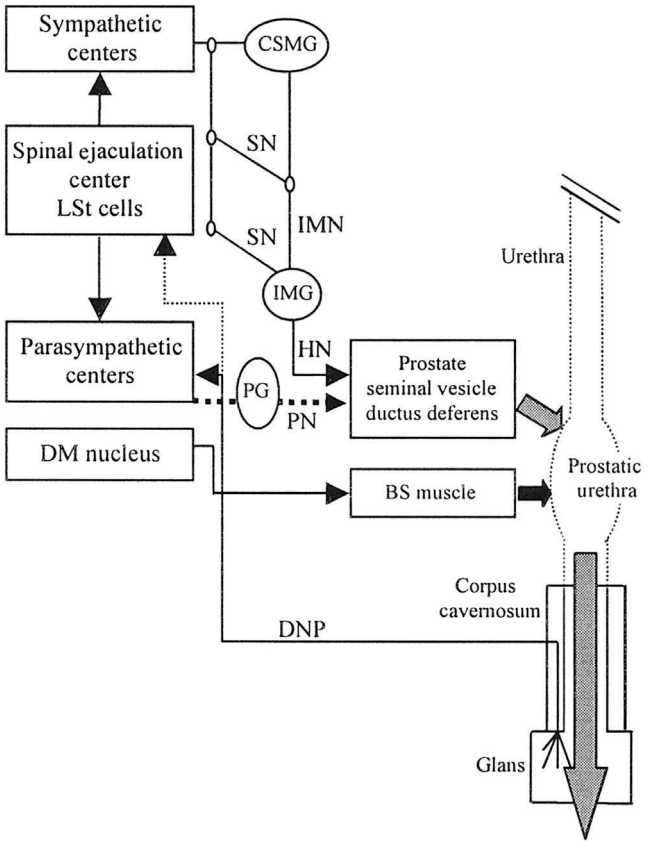

FIGURE 49.6 Schematic presentation of the spinal ejaculation generator, demonstrating the spinal cord components and autonomic, somatomotor, and sensory efferents and afferents. CSMG, coeliac superior mesenteric ganglia; IMG, inferior mesenteric ganglia; PSC, paravertebral sympathetic chain; IMN, intermesenteric nerve; HN, hypogastric nerve; PG, pevic ganglia; PN, pelvic nerve; SN, splanchnic nerve; DNP, dorsal nerve of the penis; DM, dorsomedial; BS bulbospongiosus. *Source: Reprinted from Ref. 41, with permission from Elsevier Press.*

direct stimulation of the glans. Sensory input travels via the dorsal penile nerve and pudendal nerve to the spinal cord and also via the hypogastric nerve to the paravertebral chain of sympathetic ganglia. Expulsion of semen is initiated by fibers from the ventral horn of S2-S4 spinal segments, which travel in the motor branch of the pudendal nerve to the pelvic floor muscles, including the bulbospongiosus and ischiocavernosus muscles. A nucleus in the lumbar spinal cord generates the ejaculatory response[43] (reviewed in Ref. 41).

Cellular Mediators of Erection

Both arterial and trabecular smooth muscles contribute to erection. Arterial smooth muscle relaxation increases penile blood flow, whereas trabecular smooth muscle contractions open the sinusoids in the penile erectile tissue. Trabecular smooth muscle cells have gap junctions through which electric current and second messenger signals can flow from cell to cell. Therefore, the autonomic input can synapse with relatively few cells, which in turn functionally connect in a tissue-wide network.[44]

The gaseous messenger NO is the main mediator of vascular relaxation in the corpora cavernosa (reviewed in Ref. 45). See Figure 49.7(A). NO is produced by the enzyme nitric oxide synthase (NOS), of which there are two isoforms in the penis. Neuronal, or nNOS, is in the parasympathetic nerves that innervate the erectile tissue. Endothelial, or eNOS, is in the endothelium of the erectile tissue. Parasympathetically produced NO may directly induce smooth muscle relaxation and also stimulate the production of NO by eNOS in the endothelial cells, which would further relax the penile smooth

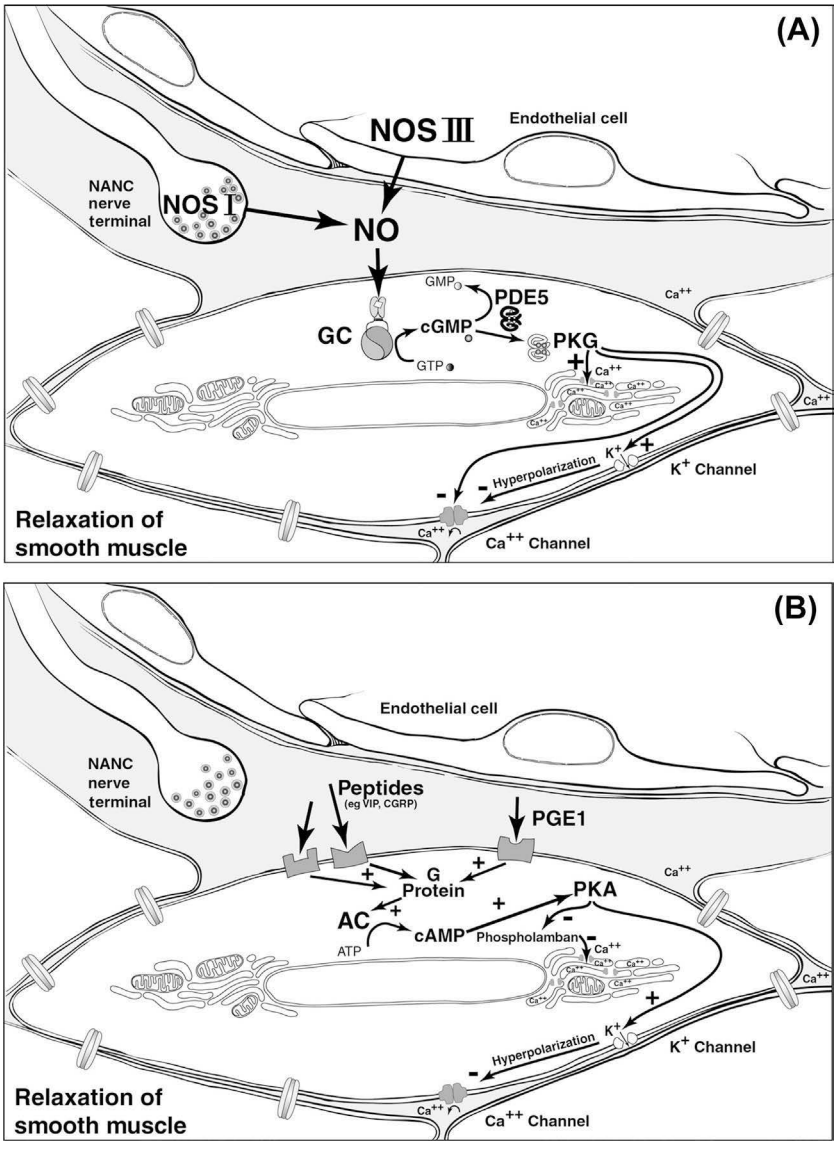

FIGURE 49.7 Mechanisms for penile smooth muscle relaxation. (A) Relaxation of penile smooth muscle: cGMP mechanisms. NO produced by nitric oxide synthase type I (NOS I) in NANC nerves and nitric oxide synthase type III (NOS III) in endothelial cells activates guanylyl cyclase (GC) in smooth muscles cells. This results in the production of cGMP from GTP. cGMP is metabolized by phosphodiesterase type 5 (PDE5). cGMP causes the activation of protein kinase G (PKG). PKG causes an increased uptake of calcium into intracellular stores and a reduction of calcium entry into the cell through calcium channels. PKG also opens potassium channels, leading to hyperpolarization, which also closes the calcium channels. The resulting decrease in intracellular calcium concentration from these mechanisms leads to relaxation of the smooth muscle. (B) Relaxation of penile smooth muscle: cAMP mechanisms. Peptides such as vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP), and prostaglandins such as PGE1, bind to specific receptors on the smooth muscle cell membrane. These are linked to G protein that activates adenylyl cyclase (AC) in smooth muscles cells. This results in the production of cAMP from ATP. cAMP causes the activation of protein kinase A (PKA). PKA causes an increased uptake of calcium into intracellular stores by inhibiting an inhibitor of calcium uptake, phospholambin. PKA also opens potassium channels, leading to hyperpolarization, which closes the calcium channels. The resulting decrease in intracellular calcium concentration from these mechanisms leads to relaxation of the smooth muscle. *Source: Reprinted from Ref. 45a, with permission from the Society for the Scientific Study of Sexuality.*

muscle.[46] Therefore, the neural stimulation engages an amplification mechanism, which greatly enhances NO release in the erectile tissue.

NO diffuses into smooth muscle cells and activates guanylyl cyclase, producing cGMP, which in turn activates protein kinase G (PKG), and to a lesser extent protein kinase A (PKA). These enzymes phosphorylate certain proteins within the cell, which ultimately results in a decrease in intracellular calcium concentration, which in turn relaxes the tissue.[47] The cGMP can relax smooth muscle in several ways: (1) activating PKG; (2) activating ion channels that sequester or extrude calcium; (3) opening potassium channels to hyperpolarize smooth muscle cells; (4) activating myosin lightchain kinases; and (5) inhibiting the contractile inositol triphosphate (IP_3) pathway.[45,48–50] Phosphodiesterase type 5 (PDE5) terminates the activity of cGMP; sildenafil prolongs cGMP activity by inhibiting PDE5.

Other processes that may contribute to erection include activation of adenylyl cyclase by vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP), and prostaglandin E_1 (PGE_1) (see Figure 49.7(B)).[51–53] These increase levels of cAMP, which activates PKA, and to a lesser extent PKG. These in turn sequester calcium and hyperpolarize the cell. At least some of the proerectile effects of PGE_1 result from an increase in NO, and repeated administration of PGE_1 increases the content of both nNOS and eNOS.[54]

Sympathetic adrenergic nerves induce and maintain detumescence via α_1 receptors on smooth muscle cells in the corpora cavernosa, which increase intracellular calcium concentration by activating the IP_3 pathway, which releases calcium from intracellular stores and allows its entry through plasma membrane calcium channels. Calcium then activates a series of events that result in contraction of smooth muscle cells, decreased blood inflow, and detumescence.[55,56] Stimulation of α_1 receptors can also increase sensitivity of smooth muscle to calcium via the Rho-kinase pathway.[57] A Rho-kinase antagonist has been shown to relax smooth muscle cells and promote erection (reviewed in Ref. 3). Therefore, antagonism of Rho-kinase may provide a new treatment for erectile dysfunction.

PRINCIPLES OF HORMONE ACTION

Male sexual behavior depends heavily on testosterone (T) and its metabolites estradiol (E2) and dihydrotestosterone (DHT). T is secreted by the Leydig cells of the testes and reaches nontesticular targets via the blood. However, the importance of gonadal steroids in male sexual behavior varies among species.[58] In most rodents, testicular steroids are essential, and castration eliminates mating. By contrast, T is more a modulatory factor for male sexual activity of humans.[59]

For most male rodents, masculinization of brain mechanisms controlling sexual behavior is primarily caused by estradiol, produced from testosterone by the enzyme aromatase (See Chapter 47). Actions via estrogen receptor α (ERα) masculinize brain circuits, whereas actions via ERβ defeminize circuits.[60] However, androgens are the masculinizing and defeminizing hormones for primates and guinea pigs (reviewed in Ref. 61). Although most aspects of sex differentiation occur perinatally, hormonal and social influences during puberty can enhance the differences that were initiated perinatally (reviewed in Ref. 62). The stimulatory effects of T on sexual behavior of adult males are termed activational effects, to distinguish them from T's organizational effects during sexual differentiation.

Genomic Effects

Because hormones circulate throughout the body, their specificity of action depends on the type of receptors in the target tissues. Most receptors that are important for sexual behavior either initiate or repress transcription of certain genes (reviewed in Refs 63,64). The steroid molecule binds to its receptor in the cytoplasm of the cell, after which the hormone-receptor complex enters the cell nucleus and dimerizes (links to another hormone-receptor complex). The dimer then binds to a hormone response element upstream of a structural gene and initiates transcription of the appropriate mRNA, which is in turn translated into a protein. That protein may be an enzyme, a receptor, or a structural protein, or it may regulate transcription of additional genes (please see Chapter 9 for more details on steroid receptor signaling). Some hormonal effects may be indirect, as a result of increased activity impinging on downstream neurons.

Besides being activated by steroid hormones, steroid receptors can be activated in a "ligand-independent" fashion (reviewed in Ref. 65), in which neurotransmitters, growth factors, peptide hormones, or intracellular messengers activate gene transcription via the steroid hormone receptors. In addition, transcriptional activity of steroid receptors can be altered by receptor coactivators and corepressors. These effects allow environmental factors to affect transcription without altering steroid hormones.

Rapid, Nongenomic Effects

Steroids may also have rapid effects via receptors on neuron membranes[66] or in an agonist-like manner at $GABA_A$ receptors,[67] thereby hyperpolarizing neurons by increasing chloride influx. Testosterone has affected neural activity in MPOA of castrated

rats within minutes[68] or in the lateral hypothalamus within seconds,[69] and estrogen or testosterone affected firing of neurons in MPOA slices within minutes.[70] However, behavioral restoration requires hours or days of steroid hormone replacement. Furthermore, rapid membrane effects may contribute to behavioral recovery, even though they are not sufficient to restore mating in castrated rats. For example, testosterone elicited activity in penile muscles within 5 min in some castrated rats, although it was not sufficient for restoration of touch-based erections at that time.[71] However, 12 or 24 h after testosterone implantation, NCE did occur, and the protein synthesis inhibitor anisomycin did not affect testosterone's activation of touch-based erections at either of those times.[72] Therefore, genomically mediated protein synthesis was not necessary for the hormonal activation of those erections.

ACTIVATION OF MALE SEXUAL BEHAVIOR BY GONADAL HORMONES

Dependence of Copulation on Recent Exposure to Testosterone

Male sexual behavior in most species is heavily dependent on hormones. Increasing production of T at puberty increases sexual activity, and sexual activity declines after castration. T is usually present in considerably greater quantities than is necessary to stimulate sexual behavior; the levels in blood reflect testicular T content, an important determinant of sperm production.[73,74] In most species, relatively small fluctuations in circulating T levels should not affect behavior.[58]

Time Course of Changes in Copulation Following Castration

Although androgens decline to unmeasurable levels within 24 h after castration,[75] male rats often continue to copulate for days or weeks.[76] In fact, in male rats the number of intromissions required to trigger ejaculation may actually decrease for a few days after castration, although intromission latencies and postejaculatory intervals increase (reviewed in Ref. 4). Therefore, one of the normal functions of T may be to increase the number of intromissions preceding ejaculation, thereby increasing the number of sperm in the ejaculate, facilitating sperm transport, and triggering a progestational state in the female.[77,78]

The postcastration decline in sexual behavior follows a sequence that is consistent across species, with loss of ejaculation first, then intromission, and later mounting (reviewed in Ref. 3). The differential loss of copulatory elements after castration is due in part to their different peripheral target cells or mechanisms. For example, mounting does not require tactile sensitivity of the penis or its ability to become erect, as intromission does, and ejaculation requires even more penile sensory input and motor output than does intromission. The various behaviors may also depend on different neuronal circuits, which have different degrees of hormonal dependence.

There are mixed reports of the effects of castration on sexual desire and copulation in men. Kinsey[79] relied on anecdotal accounts to conclude that the assertion that castration impaired sexual function in most men had little basis. Subsequently, more detailed reports revealed considerable variability. Heim and Hursch[59] reviewed the results of prospective studies of men who had been castrated as "treatment" for sexual offenses. Half to two-thirds of those men reported a rapid loss of sexual desire and interest, whereas sexual activity in the remaining men waned gradually, with as many as 10% reporting sexual intercourse for up to 20 years. There was a greater effect the older the individual was at the time of castration.[59] Imaging studies revealed that visual sexual stimuli activated regions of men's brains that control autonomic and neuroendocrine functions, and this activation was correlated with testosterone levels.[80] In imaging studies of hypogonadal men some of those same regions showed less activation, compared to eugonadal men, whereas hormone replacement therapy led to partial recovery of stimulus-induced brain activation.[81] Finally, hypogonadal patients reported recovery of erectile activity after testosterone treatment, as indicated by nocturnal erections, similar to levels observed in eugonadal men.[82]

Time Course of Changes in Copulation Following T Restoration

Exogenous testosterone restores copulatory behaviors in the reverse order in which they were lost, with mounting occurring first, followed by intromission, and finally ejaculation.[83] In long-term castrates, 5–10 days of T exposure is usually required to reinstate copulation.[76,84–86] However, T increased neural firing in the MPOA within minutes[68] and increased firing in the lateral hypothalamus within seconds.[69] Furthermore, E stimulated chemoinvestigation and mounting in castrated rats within 35 min,[87] and in castrated mice T facilitated mounting within 60 min.[88] Therefore, steroids begin to act immediately within the brain, but longer-term genomic effects are necessary for complete restoration of mating. In support of this, the protein synthesis inhibitor anisomycin blocked the effects of T on copulatory behavior.[89] However, T restoration of touch-based erections was not disrupted by anisomycin, and in some conditions anisomycin actually facilitated restoration of those erections.[72]

The Amount of Testosterone Required for Restoration

Higher doses of hormone are necessary to restore copulation in long-term castrates than in those receiving hormone soon after castration.[76] Apparently, continuous exposure of neural and peripheral tissues to T maintains tissue responsiveness, whereas reactivation of dormant copulatory systems requires greater hormonal input. Furthermore, the dose of T influences the degree of recovery. Rat castrates that received a high dose of T, which restored normal physiological levels, experienced the greatest recovery, compared to those receiving vehicle or low and medium doses of T.[90] After 16 days of hormone replacement, 100% of those receiving the high dose could ejaculate, whereas no vehicle-treated animal ejaculated, and 30% and 80% of those receiving low and medium doses, respectively, displayed ejaculations.[90] However, individual males vary widely in their copulatory abilities, and once an individual's pre-castration ability is reached, additional T treatment produces little benefit.[91,92]

The Role of T Metabolites in Maintaining and Restoring Copulation

T is the principal steroid hormone produced by the testes. However, T is converted in target organs to potent estrogenic or androgenic metabolites. Estradiol (E2), formed by the P450 enzyme aromatase, binds to ERs, of which there are at least two types: ERα and ERβ. The major androgenic metabolite of T is DHT, which is formed by the enzyme 5α-reductase. Both T and DHT bind to the androgen receptor (AR), but DHT binds with approximately fivefold greater affinity.[93] Furthermore, DHT cannot be aromatized to E2, and therefore is considered to have a "pure" androgenic action. Some target cells may produce both E2 and DHT and have both ERs and ARs.

Estrogens and DHT have differential effects on copulation. In male rats E2 is the main steroid that maintains or restores most copulatory elements, and systemic aromatase inhibitors or ER antagonists inhibit restoration of copulation by testosterone (reviewed in Ref. 4). Furthermore, synthetic androgens (5α-androstanediols) that can be aromatized to E2, but not 5α-reduced to DHT, are even more effective than T in restoring sexual behavior in castrated rats[94] or mice.[95] Similar inhibitory effects of an aromatase inhibitor are found in castrated, T-treated monkeys.[96] Furthermore, plasma concentrations of E2 and of the 5-HT precursor tryptophan, but not of T or DHT, are correlated with the ability of male macaques to copulate to ejaculation; however, sexual motivation is more related to androgen concentrations.[97] The effectiveness of E2 in restoring or maintaining copulation, and the lack of effectiveness of DHT and other nonaromatizable androgens, in several rodent species is similar to the organizational "aromatization hypothesis". That is, the aromatization of T to E2 is the critical step in the masculinization of the brain in early development.

However, E2 alone cannot maintain full copulatory behavior. Frequently, E2-treated castrates are unable to ejaculate or have fewer ejaculations than males treated with T or with a combination of E2 and DHT.[98,99] Furthermore, E2 alone did not restore partner preference, and the aromatase inhibitor fadrozole did not block T's restoration of partner preference [99]. A similar lack of effect of fadrozole was observed in male hamsters.[100] However, a recent study of male Syrian hamsters reported that co-administering DHT with T restores sexual behavior more rapidly than does T alone, suggesting an additive interaction between DHT and T.[101] The steroidal ER antagonist RU-58,668 also does not block T's restoration of copulation or partner preference in male rats, but does inhibit T's restoration of scent marking.[102] Therefore, activation of estrogen receptors is not always sufficient to stimulate copulation or partner preference in male rats or hamsters, and ER antagonists do not always render testosterone ineffective.

Also supporting the role of androgens in rodents, the antiandrogen flutamide reduces T's restoration of copulation, partner preference, scent marking, and 50 kHz ("attraction") vocalizations in castrated rats.[102] An androgen antagonist with greater affinity for the androgen receptor (SCH-16423) eliminates copulation in most castrates treated with a dose of T that restores ejaculation in all control males.[103]

Androgens may be more important for the appetitive aspects of sexual behavior than for copulation per se. DHT treatment in castrated rats stimulates NCE and ultrasonic vocalizations, but E2 does not.[104,105] Similarly, flutamide impaires T's restoration of partner preference, scent marking, and 50-kHz vocalizations in castrated rats, while the ER antagonist RU-58,668 inhibits only scent marking and ultrasound.[102] In related studies, the aromatase inhibitor fadrozole does not prevent partner preference in castrated rats with T replacement.[99] Fadrozole does reduce anticipatory level searching for a female in a bilevel motivation test, but E2 treatment only partially restores the behavior.[106] Taken together, these studies suggest that stimulation of ER is not sufficient, and in some cases may not be necessary, to restore or maintain full copulatory behavior in male rats or Syrian hamsters.

Studies of genetically altered mice lacking either ERα or ERβ have furthered our understanding of the roles of androgens and estrogens in male sexual activity. Both ERα and ERβ are normally expressed in the brain.[107] Gonadally intact male mice lacking the ERα (ERα "knockout" mice, or ERαKO) mount normally, but have fewer intromissions than wild-type mice, and almost no ejaculations.[108,109] This is not due to endocrine dysfunction, because ERαKO males secrete more T than

do wild-type mice, because of decreased ER-mediated negative feedback of E2 on LH secretion.[110] Furthermore, castration of ERαKO males and replacement with normal levels of T[110] or with higher than normal levels of DHT[109] increase mounting, but do not restore ejaculation. However, concurrent treatment with T and a DA agonist can activate masculine sexual behavior in ERαKO males.[111] AR immunoreactivity[108] and ERβ mRNA[112] are comparable in ERαKO and wild-type mice, indicating a lack of compensatory upregulation. Deficits in sexual behavior have also been reported in aromatase knockout (ArKO) mice.[113,114] Although ArKO mice can sire litters when housed with females, they are less able to discriminate odors from conspecifics.[113] In contrast to the dramatic effects of ERαKO or ArKO on male sexual behavior, there are no deficits in adult mating behavior of ERβ knockout (βERKO) mice,[115] although βERKO males have a later onset of ejaculation at puberty. Therefore, ERα and aromatase, perhaps in concert with androgens, appear to play a substantial role in male sexual behavior in mice.

In a number of species, the aromatization hypothesis has little or no support. DHT can maintain or restore copulation in male rabbits, guinea pigs, hamsters, deer mice, monkeys, mice, and humans, but not in gerbils, pigs, or sheep (reviewed in Ref. 3). In ferrets, either T or E facilitate partner preference in sexually experienced males,[116] and E2 appears to contribute to libido in dogs[117] and to sexual activity in monkeys.[96] However, although the optimal balance of androgenic and estrogenic stimuli may vary among species, males are normally exposed to both androgenic and estrogenic metabolites of T, and both may contribute in some way to mating.

Effects of Hormone Deprivation and Replacement on *Ex-Copula* Penile Responses

Animal Studies

Touch-based and NCE are lost more rapidly after castration (2–3 days) and are also restored more rapidly (2–3 days) by exogenous T than is copulation (5–7 days) (reviewed in Ref. 3). Therefore, the hormonal stimulation of noncontact and touch-based erections may differ from hormonal control of copulation, at least regarding temporal factors.

Another difference in hormonal control of penile reflexes, compared to copulation, is the lack of effect of E2, and the effectiveness of DHT, in restoring or maintaining touch-based or NCE in rats (reviewed in Refs 3,4). The DHT regimens that maintain or restore penile reflexes are not effective in copulation tests. Therefore, as with temporal factors, the hormonal mechanisms that control *ex-copula* erections are different from those that regulate copulation.

Although E2 is not effective in *ex-copula* reflex tests, it can maintain erections during copulation. Also, the duration, frequency, and average amplitude of EMG responses of the bulbospongiosus muscle are at least as great in E2-treated castrates as in T-treated castrates.[118] Sachs[119] suggested that a copulatory "behavioral cascade" is organized in the brain and activates reflexes that are observable only in copulatory contexts. Reflexive erections may be elicited primarily by disinhibition of lumbosacral spinal circuits. Although E2 cannot disinhibit reflexes *ex copula*, it apparently can activate those same reflexes during mating.

Studies on Human Males

Testosterone concentrations in men with erectile dysfunction usually differ little from those of normally functioning men.[120,121] However, exogenous T does improve erectile function in aging men with moderate decreases in T.[122,123] Also, administration of T to hypogonadal men (which results in normal levels) or to eugonadal men (which produces supraphysiological levels) does increase arousal in response to sexual audiotapes.[124] T and DHT are equally effective in agonadal men, and neither an ER antagonist nor an aromatase inhibitor inhibits T-induced sexual function. Thus, androgenic stimulation in men can facilitate sexual interest and ability (reviewed in Ref. 125).

Men with low to normal T concentrations, given short-term T replacement together with sildenafil, experience increased arterial inflow to the penis.[126] Furthermore, hypogonadal men can have erections stimulated by fantasy or viewing erotic films; their loss of erection is restricted to nocturnal and spontaneous erections.[127] Thus, the effect of androgen on erection in men is context-sensitive, as it is in rats.

EFFECTS OF SYSTEMICALLY OR INTRACEREBROVENTRICULARLY ADMINISTERED DRUGS

Sexual behavior requires rapid sensory and motor processing and complex moment-to-moment interactions with a partner. Consequently, the slower effects of hormones must be translated into more rapid neural responses. Hormones bias sensory inputs to favor processing of sexually relevant stimuli and also prime central integrative areas and motor outputs to promote appropriate sexual responses. Major targets of steroid priming are enzymes that synthesize (or catabolize) neurotransmitters or neuromodulators, mechanisms that govern neurotransmitter release, receptors, or second messenger systems.

Dopamine

DA has long been known to facilitate sexual function. Administration of L-DOPA to Parkinsonian patients often results in increased libido and sexual potency,[128,129] and the classic DA agonist apomorphine has been used

to treat erectile dysfunction.[130–132] A new sublingual form of apomorphine appears to be clinically effective, with fewer side effects than subcutaneous injections of apomorphine, such as increased libido or nausea.[133,134]

In rats, systemically administered DA agonists have also facilitated male sexual behavior (reviewed in Refs 134–136). L-DOPA and/or apomorphine can induce sexually sluggish males to copulate, increase the numbers of ejaculations, and decrease ejaculation latency and the numbers of intromissions preceding ejaculation. Apomorphine can also elicit copulation in sexually satiated male rats[137,138] and in socially stressed mice[139] and rats.[140] In male rats that were sexually sluggish because of medial frontal cortex damage, DA agonists have decreased their usual long mount and intromission latencies,[141] and in short-term[142] and long-term[143] castrated rats, apomorphine has partially restored copulation. Mice lacking the Esr1 gene, which codes for ERα, usually show little copulatory behavior, yet they can copulate normally after receiving systemic injections of apomorphine.[111] Therefore, stimulation of DA receptors appears to render both organizational and activational effects of ERα unnecessary for male copulatory behavior, at least in some animals. Systemic administration of apomorphine also elicits penile erections and genital grooming in mice.[144] Its effects are blocked by the centrally active antagonist haloperidol, but not by domperidone, which does not cross the blood–brain barrier. Cocaine, which inhibits DA reuptake and thereby increases synaptic DA, increases the percentage of rats that copulate and produces additive effects with paradoxical sleep deprivation, which the authors suggest may increase sensitivity of DA receptors.[145] Apomorphine administered systemically to hamsters has produced only slight decreases in ejaculation latency and postejaculatory interval, although it has also abolished long intromissions (a sign of approaching sexual satiety) in 60% of the animals.[146]

Daily injections of amphetamine, which releases endogenous DA, result in increased locomotor responsiveness to amphetamine (sensitization). Similar injections in sexually naive male rats facilitate copulation on their first test.[147] Therefore, the presumably dopaminergic mechanisms that result in drug sensitization also "cross-sensitize" to a natural motivated behavior. Furthermore, similar mechanisms may at least partially explain the enhanced copulatory ability and resistance to surgical and environmental insults of sexually experienced animals.

As expected, antagonists that block the effects of DA interfere with sexual behavior of experienced and inexperienced male rats (reviewed in Ref. 3). The effects of DA antagonists range from failure to copulate to increased latencies to intromit and ejaculate. The numbers of touch-based erections are also decreased by a centrally active antagonist, haloperidol, but not by an antagonist that does not cross the blood–brain barrier, domperidone.[148]

The DA antagonist haloperidol inhibits measures of sexual motivation, as well as copulation. Sexually naive male rats were given 10 daily trials on which they traversed a runway to be near an estrous or a nonestrous female on different days.[149] They were then injected with vehicle or one of three doses of haloperidol and allowed to copulate to one ejaculation; no copulatory measure was significantly affected by the drug. Two days later vehicle-treated animals ran faster after sexual experience than before and also ran faster for the estrous than for the nonestrous female. However, haloperidol dose-dependently decreased running speed and preference for the estrous female. Haloperidol-treated males also showed more retreats as they approached the goal box, as if it were aversive. The drug was given two days before the runway test and had sufficient time to be metabolized before that test. Also, no dose significantly affected copulation on the experience day. Thus, a systemically administered DA antagonist selectively impaired the experience-induced enhancement of sexual motivation.

A similar experiment from the same lab supported their earlier conclusion. Two doses of haloperidol or vehicle were given to sexually naive males before traversing a runway for an empty goal box, an estrous female, or a nonestrous female, following a 4-min exposure to the target through a perforated barrier.[150] Run times for the empty goal box and for the nonestrous female were not affected by the drug, suggesting that motoric ability was not compromised. However, run times for the estrous female were slower, compared to vehicle-treated animals, and were not significantly different from their own run times to the other two targets.

DA receptors are classified into two families. The D_1 family comprises D_1 and D_5 receptors, while the D_2 family consists of D_2, D_3, and D_4 receptors. The D_1-like receptors stimulate adenylyl cyclase, whereas the D_2 family members inhibit adenylyl cyclase and also influence certain ion channels and the phosphoinositide system. DA and apomorphine activate both families, but drugs that are relatively selective for a given subtype have been used to analyze the roles of the subtypes in various aspects of copulation.

There is some agreement that stimulation of D_2-like receptors facilitates ejaculation. A D_3-selective agonist (7-OH-DPAT) and two D_2/D_3 agonists (SND 919 and B-HT 920) decreased ejaculation latencies and the numbers of intromissions that preceded ejaculation in sexually active male rats, but not in sexually inactive animals.[151–153] Conversely, a D_2/D_3 antagonist (eticlopride) blocked the pro-ejaculatory effects of 7-OH-DPAT[154] and of SND 919,[155] confirming their receptor specificity. However, cholinergic muscarinic receptors may mediate

the pro-ejaculatory effects of D_2-like receptors, as the muscarinic antagonist atropine inhibited the effects of both dopaminergic and muscarinic agonists.[156] The effects of dopaminergic agonists are dose dependent, with low doses facilitating and high doses inhibiting copulation, probably as a result of stereotypic behaviors elicited by the high doses.[157] A D_1 agonist (SKF 81292) facilitated mounting in genetically DA-deficient mice, whereas a D_2 agonist (quinpirole) inhibited mounting as it increased stereotypic behavior.[158] A different D_1 agonist (SKF 38393) also increased the time spent in a goal compartment with a receptive female.[159] The drug increased the number of copulatory behaviors but did not affect motoric measures. Thus, a measure of sexual motivation was again affected independently of obvious motor confounds.

There are apparently contradictory effects of D_1- and D_2-like agonists on *ex-copula* genital reflexes. In some experiments, a presumed-selective D_2 agonist elicited erections in a neutral arena, whereas a D_1 agonist inhibited such erections; however, a D_2/D_3 antagonist administered with a presumed-selective D_2 agonist actually increased drug-induced erections, suggesting that stimulation of D_2-like receptors may inhibit, rather than stimulate, erections (reviewed in Ref. 3). It is likely that the presumed-selective D_2-like agonists also had substantial activity at D_1 receptors. D_2-like receptors may also inhibit touch-based erections in restrained male rats. A D_2/D_3 agonist (quinelorane) decreased touch-based erections following either systemic or intra-MPOA administration.[160] As will be discussed below in the section on the MPOA, intense stimulation of D_2-like receptors appears to shift autonomic balance to favor sympathetically mediated seminal emission and ejaculation, and away from parasympathetically mediated erection. However, two selective D_2/D_3 agonists (7-OH-DPAT and B-HT 920) elicited erections, as well as stretching, yawning, and sedation in sexually naive male rats in a neutral arena.[161] It is not clear whether different dose ranges or different test regimens (touch-based erections versus drug-induced erections in a neutral arena) may account for these divergent findings.

The facilitative influence of D_1–like receptors on male sexual behavior appears to be conserved across phyla. Systemic administration of a D_1 agonist (SKF 81297) to two species of lizards increased the proportion of animals that mated after gonadectomy and decreased mount latencies.[162] Conversely, systemic injections of a DA receptor antagonist in leopard geckos inhibited courtship behavior in castrated, T-implanted males (reviewed in Ref. 163). Similarly, in quail systemic injections of a D_1 agonist (SKF 38393) increased two measures of appetitive behavior: the amount of time near a window through which a female could be seen and the time looking through the window; it also increased two copulatory measures: the numbers of mount attempts and of cloacal contact movements.[164] Conversely, a D_1 antagonist (SCH 23390) decreased the approaches to the female's window and the number of mount attempts. Administration of D_2-selective drugs produced the opposite pattern of results: the D_2 agonist (quinpirole) decreased both appetitive and copulatory measures, and a D_2 antagonist (spiperone) increased mount attempts but decreased the number of times the male approached the area of the window. An earlier article reported that the classic DA agonist apomorphine inhibited both appetitive and copulatory measures, apparently by stimulating primarily D_2 receptors.[165] The inhibition by the two higher doses was associated with intense stereotyped pecking; however, the lowest dose inhibited sexual behavior without significant increases in pecking. Two indirect DA agonists (nomifensine, which inhibits reuptake, and amfonelic acid, which primarily increases release) produced mixed facilitative and inhibitory effects. A more recent article from the same lab reported that intracerebroventricular (ICV) injections of DA itself inhibited sexual behavior in quail, and suggested that alpha-2 noradrenergic receptors, rather than DA receptors, mediated the inhibitory effects.[166] However, the earlier studies using selective DA agonists and antagonists probably avoided the problem of cross-reactivity with alpha-2 receptors.

In summary, there is impressive agreement that systemically administered DA agonists facilitate several aspects of mammalian sexual behavior, including sexual motivation, genital reflexes, and copulation itself, and that DA antagonists inhibit those same measures. Systemically administered DA agonists have restored sexual behavior in castrates, in stressed or sexually satiated males, in males with prefrontal cortex lesions, in males lacking ERα, and in males lacking endogenous DA (reviewed above). DA agonists have also facilitated erections in several contexts, although the relative importance of D_1- and D_2-like receptors for genital reflexes is not clear. Conversely, DA antagonists have inhibited both conditioned and unconditioned sexual motivation, in several cases without confounding motor effects; have decreased genital reflexes; and have decreased the incidence, rate, and efficiency of copulation. There is also conservation of the facilitative role of D_1-like receptors in reptiles, birds, and mammals.

A critical review has suggested that DA has no importance for sexual motivation, because several of the studies that reported inhibition by DA antagonists either used measures of approach to a female, which can confound motoric and motivational factors, or studied sexually experienced males.[167] However, several of the studies using approach to a female specifically ruled out motoric effects, as noted above. Furthermore, the increase in sexual motivation that follows copulatory experience is an

important issue, and experiments have separately studied dopaminergic effects on both the initial conditioning and the subsequent expression of sexual motivation. DA antagonists have inhibited both the initial sensitization and the subsequent expression of the enhanced motivation, without confounding motor effects. While no procedure has been devised to measure motivation selectively, without any possibility of sensory, learning, or motoric contributions, the preponderance of evidence suggests that DA contributes to sexual motivation, as well as to genital reflexes and copulatory performance.

Norepinephrine

Experiments using noradrenergic drugs and lesions of NE pathways have produced inconsistent results. Electrolytic lesions of the ascending dorsal noradrenergic bundle in the midbrain, which destroy both cell bodies and fibers of passage, resulted in decreased postejaculatory intervals and increased ejaculations in a 1-h test.[168,169] However, cytotoxic (6-OHDA) lesions of the same area were without effect on copulation.[169] Electrolytic lesions of the locus coeruleus or systemic inhibition of NE synthesis inhibited copulation in one experiment[170] but not in a more recent one.[171] However, lesions of the locus coeruleus blocked the "Coolidge effect"(the facilitative effect of introduction of a new female to a sexually satiated male) and decreased the facilitative effects of 8-OH-DPAT, a 5-HT_{1A} agonist in satiated and nonsatiated males.[171]

Presynaptic α_2 autoreceptors exert inhibitory control of NE release. Inhibition of α_2 autoreceptors with yohimbine can increase mounts and intromissions by sexually sluggish males,[172,173] castrated males,[174] and males with anesthetized penes.[172] Yohimbine can also reverse sexual satiety.[138,175,176] However, yohimbine's effects on satiety were inhibited by the DA antagonist haloperidol, suggesting that activation of the DA system mediated yohimbine's effects.[138] A more selective α_2 antagonist (delaquamine) can also facilitate copulation in sexually naive males and increase mounting by castrates.[177] Copulatory behavior was facilitated by two other α_2 antagonists; rauwalscine decreased the intercopulatory and postejaculatory intervals, and phentolamine decreased the number of intromissions and time preceding ejaculation.[178] In hamsters, too, yohimbine appears to facilitate copulation, increasing the number of ejaculations and the numbers of long intromissions (usually a sign of approaching satiety).[146] Therefore, increasing NE release by blocking α_2 autoreceptors appears to enhance sexual performance in both rats and hamsters. Conversely, stimulating α_2 autoreceptors with clonidine can inhibit copulation and touch-based *ex-copula* erections in rats.[179] However, high doses of yohimbine inhibited copulatory behavior,[180] and the NE precursor dl-threodihydroxyphenylserine increased mount and intromission latencies.[141] Therefore, increased NE activity may increase or decrease sexual behavior. Moderate doses of yohimbine may facilitate sexual behavior by increasing NE release and thereby stimulating α_1 receptors; blocking α_1 receptors with prazosin or methoxamine can impair copulation.[181] Yohimbine also facilitates erections in men with moderate erectile dysfunction, with greater success in men with less severe dysfunction and higher levels of T.[182]

As the major postganglionic neurotransmitter of the sympathetic nervous system, NE promotes penile detumescence and inhibits penile reflexes. In contrast to its facilitation of copulation, yohimbine inhibits *ex-copula* touch-based erections and seminal emission.[183] However, the α_2 agonist clonidine also inhibits touch-based erections.[179] Because both stimulation and inhibition of α_2 receptors can decrease penile reflexes, it appears that low to moderate levels of NE may facilitate erections, whereas high levels are inhibitory.

Systemically administered drugs affect both central and peripheral sites that control genital reflexes. However, guanethidine, which depletes peripheral noradrenergic nerve terminals without affecting central sites, has been used to separate these influences. A single injection increased the number of anteroflexions ("flips") in restrained supine males; however, treatments over 4–8 weeks decreased touch-based erections and especially seminal emissions.[184] Therefore, there appears to be a facilitative influence of the sympathetic nervous system on erection, as also demonstrated by Giuliano and colleagues,[38] in addition to its major role in seminal emission. The mixed excitatory and inhibitory influences of NE on male sexual behavior may be relevant to human sexual function, as reviewed insightfully by Bancroft & Janssen.[185]

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is generally inhibitory to sexual behavior. A common side effect of selective serotonin reuptake inhibitor (SSRI) antidepressants is difficulty achieving orgasm or ejaculation, accompanied sometimes by decreased sexual interest (reviewed in Ref. 186). These inhibitory effects typically increase over the same time course as the antidepressant effects. The physiological mechanisms that mediate these effects are unclear, but they may include increased prolactin secretion, anticholinergic effects, decreased NO production,[186] or decreased penile sensitivity.[187] SSRIs have also been used to treat premature ejaculation (reviewed in Ref. 3), although unwanted side effects may include anorgasmia and decreased sexual interest.[188]

Chronic administration of the SSRI fluoxetine to rats has also resulted in decreased sexual motivation and

ejaculation (reviewed in Ref. 3). Injections of the neuropeptide oxytocin (see below) can reverse fluoxetine's inhibition of ejaculation but not of sexual motivation, measured as level changes in a bilevel apparatus, in which the male climbs from one level to a higher one, or descends back to the lower level, in search of a female.[189] Therefore, one possible mechanism for fluoxetine's inhibition of ejaculation may be a decrease in oxytocin in some area. In hamsters, too, a 5-HT reuptake inhibitor, clomipramine, administered for 14 days in sugar water, decreased the number of ejaculations.[190] One mechanism of the inhibition of ejaculation may be a decreased pressure response of the seminal vesicle to splanchnic nerve stimulation.[191] Ejaculation was not inhibited in most studies of acute, as opposed to chronic, administration of fluoxetine.[189,192] However, in one study acute injections slowed copulation, an effect that was reversed by amantadine, an NE releaser, suggesting an interaction between 5-HT and NE influences.[193] Acute injections of fluoxetine inhibited one measure of sexual motivation (level changing in search of a female) but not time spent near a receptive female.[194] Injections of the 5-HT precursor, 5-hydroxytryptophan, also increased the latencies to intromit and ejaculate and increased the intromissions preceding ejaculation.[195] Similarly, administration of 5-HT intrathecally (around the spinal cord) abolished the urethrogentital reflex, which was restored by systemic injections of methysergide, a 5-HT antagonist.[196]

Copulation has been facilitated by drugs that deplete 5-HT and by lesions of the medial, but not the dorsal, raphe nucleus (reviewed in Ref. 3), a major source of 5-HT in the brain. Facilitative effects are found most often in animals that were originally noncopulators, in sexually naive males, or in castrates maintained on suboptimal T replacement (reviewed in Ref. 3). Similarly, 5-HT depletion resulting from lesions of the median and pontine raphe nuclei has increased intense touch-based penile erections (cups) and anteroflexions,[197] and neurotoxic lesions of 5-HT terminals in the brain and spinal cord have disinhibited the UG reflex.[198] Inhibition of 5-HT synthesis by p-chlorophenylalanine (pCPA) decreased the latency to the first touch-based erection, but also decreased the numbers of erections.[199] pCPA-treated males were more likely to have NCE in the presence of a female, but not when tested alone.[199] Thus, 5-HT inhibits erections in response to a female, and has mixed, but mostly inhibitory effects on touch-based erections.

In contrast to 5-HT's generally inhibitory effects on sexual behavior, stimulation of one receptor subtype, the 5-HT_{1A} receptor, facilitates ejaculation. The classic 5-HT_{1A} agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) has decreased both the time and number of intromissions preceding ejaculation, with some rats ejaculating on their first intromission (reviewed in Ref. 3). Flesinoxan, another 5-HT_{1A} agonist, has also facilitated ejaculation, although no animals ejaculated on their first intromission.[200] Because the effects of 8-OH-DPAT are opposite those of 5-HT itself, and because some 5-HT_{1A} receptors are autoreceptors on serotonergic somas and dendrites, it was initially thought that 8-OH-DPAT facilitated sexual behavior by inhibiting 5-HT release. However, lesions of serotonergic nuclei did not alter the effects of 8-OH-DPAT, suggesting that postsynaptic receptors mediated its effects.[201] In addition, microinjections of the drug into terminal fields (MPOA and nucleus accumbens), where all 5-HT_{1A} receptors are postsynaptic, also facilitated sexual behavior.[202,203] The effects of 8-OH-DPAT are dependent on normal levels of T and on sexual experience. In castrates with subnormal T replacement, 8-OH-DPAT did not facilitate copulation, and in those with threshold T levels, only sexually experienced males copulated after 8-OH-DPAT administration.[204] Males with high levels of T showed a strong effect of the drug.

A likely mechanism of 8-OH-DPAT's effects is an increase in noradrenergic activity in some brain area(s). 8-OH-DPAT, the α_2-adrenergic receptor antagonist yohimbine, and the opioid antagonist naloxone all increased the percentage of sexually satiated males that copulated on the following day.[175,176] Furthermore, neurotoxic lesions of noradrenergic neurons blocked the facilitation by 8-OH-DPAT and naloxone in satiated males, but not that of yohimbine.[176] Similar lesions also decreased the effects of 8-OH-DPAT in non-exhausted males.[171]

The most common effect of 8-OH-DPAT is facilitation of ejaculation, which is mediated in part by the sympathetic nervous system. In agreement with a prosympathetic, anti-parasympathetic influence, 8-OH-DPAT inhibited touch-based erections, cups, and anteroflexions in male rats, in addition to decreasing the time and number of intromissions preceding ejaculation.[205] A moderate dose of 8-OH-DPAT also decreased NCE[206] and facilitated ejaculation in monkeys, although a high dose delayed ejaculation.[207] Thus, stimulation of 5-HT_{1A} receptors appears to shift autonomic balance to favor ejaculation, in addition to promoting copulation in sexually inactive males. However, the effects of 8-OH-DPAT show some species specificity. Low doses had no effect in male ferrets, and the same doses that facilitated ejaculation in male rats inhibited copulation in ferrets.[208]

Stimulation of other 5-HT receptor subtypes results in generally inhibitory effects on copulation, as expected from the inhibitory effects of 5-HT itself. In rats the 5-HT_{1B} agonist anpirtoline inhibited ejaculation; its effects were blocked by two selective 5-HT_{1B} antagonists.[209] Co-administration of an SSRI (citalopram) and a 5-HT_{1A} antagonist lengthened ejaculation latency, and that effect was blocked by a 5-HT_{1B} antagonist.[210]

A 5-HT_{1B} antagonist also blocked the increased ejaculation latency resulting from administration of the 5-HT precursor 5-hydroxytryptophan (plus a peripheral decarboxylase inhibitor to restrict the increase in 5-HT to the CNS).[211] 5-HT_{1B} knockout mice were less debilitated by several serotonergic drugs than were wild-type mice, again suggesting an inhibitory effect of the 5-HT_{1B} receptor[212] Consistent with a facilitative effect of 5-HT_{1A} receptors on ejaculation, a 5-HT_{1A} antagonist, co-administered with the SSRI, produced a further lengthening of ejaculation latency. Therefore, 5-HT_{1A} and 5-HT_{1B} receptors have opposite effects on ejaculation, with 5-HT_{1A} receptors facilitating ejaculation and 5-HT_{1B} receptors inhibiting it.

In contrast to the opposing effects of 5-HT_{1A} and 5-HT_{1B} receptors in rats, two studies of mice reported inhibitory effects of both the 5-HT_{1A} agonist 8-OH-DPAT and two 5-HT_{1B} agonists. The 5-HT_{1A} agonist 8-OH-DPAT decreased both the time spent near a receptive female[213] and copulatory performance.[212] The 5-HT_{1B} agonist CGS-12066A also decreased time spent near a receptive female,[213] and the 5-HT_{1B} agonist TFMPP inhibited the execution of copulation. On the other hand, 5-HT_{1B} knockout mice required more stimulation to achieve ejaculation, but were less sensitive to the inhibitory effects of 5-hydroxytryptophan, the 5-HT precursor.[212] The authors suggested that compensatory mechanisms may have rendered the knockout mice less sensitive to the inhibitory effects of high levels of 5-HT.

Stimulation of 5-HT_{2C} receptors may have effects opposite those of 5-HT_{1A} agonists: facilitation of erection and inhibition of ejaculation. The 5-$HT_{2C/1D}$ agonist mCPP increased the number of male monkeys that had drug-induced and NCE, but decreased the number of males that initiated copulation and achieved ejaculation.[206 a] mCPP has also increased firing of the cavernous nerves and increased intracavernous pressure in anesthetized rats[214] and resulted in drug-induced erections in awake rats.[215] A more selective 5-HT_{2C} agonist (RO60-0175) also increased erections in awake rats.[216] Therefore, 5-HT_{1A} receptors appear to promote sympathetically mediated ejaculation, whereas 5-HT_{2C} receptors facilitate parasympathetic effects on erection, but may inhibit ejaculation.

A relatively nonselective 5-HT_2 agonist, DOI, has inhibited copulation, and a 5-HT_2 antagonist facilitated copulation (reviewed in Ref. 3). To summarize, 5-HT_{1B}, 5-HT_{2C}, and other 5-HT_2 agonists inhibit ejaculation and may impair copulation, although 5-HT_{2C} agonists may also promote erection. The 5-HT_{1A} agonists, on the other hand, facilitate ejaculation and, in otherwise inactive males, stimulate copulation.

Acetylcholine

Acetylcholine (ACh) is produced from choline and acetyl coenzyme A by choline acetyltransferase. Eight major groups of cholinergic cell bodies (Ch 1–8) lie in the basal forebrain and brainstem. ACh is also the neurotransmitter for peripheral motor nerves, preganglionic autonomic neurons, and postganglionic parasympathetic neurons. There are five types of muscarinic receptors, coupled to either $G_{q/11}$ or $G_{I/O}$ G-proteins. Their effects may be either excitatory or inhibitory, depending on the tissue.[217] Nicotinic receptors have been divided into nine subtypes and are excitatory, acting on sodium channels.[217]

Increasing levels of ACh by slowing its degradation (using the acetylcholinesterase antagonist physostigmine) or administration of the muscarinic agonist pilocarpine increased the number of ejaculations; these effects were blocked by the muscarinic antagonist atropine.[156] Pilocarpine also induced erections in a neutral chamber.[218] The muscarinic agonist oxotremorine also facilitated copulation in both experienced and naive castrates with T replacement, but not in males without T.[219] Therefore, systemic administration of muscarinic drugs appears to facilitate copulation and *ex-copula* erections. However, because systemically administered cholinergic drugs affect striated muscles and parasympathetic targets, it is difficult to discern whether their effects are centrally or peripherally mediated.

Gamma-Amino Butyric Acid

Gamma-amino butyric acid (GABA) is the most common inhibitory neurotransmitter in the brain. Systemic injections of both $GABA_A$[220] and $GABA_B$[221] agonists impaired copulation without effects on motor activity. Similar effects of the $GABA_A$ agonist were seen in rabbits.[222] However, a different $GABA_A$ agonist, as well as an inhibitor of GABA synthesis and an inhibitor of GABA catabolism, all impaired social and drinking behaviors, as well as sexual behavior, suggesting that these treatments had nonspecific effects on motivated behaviors.[223] Furthermore, some inhibitory effects on copulation may have been mediated by decreased EMG activity in ischiocavernosus muscles during thrusting, a peripheral, rather than central effect.[224]

Opioids

Opiates and endogenous opioids have mixed, dose-dependent effects on male sexual behavior (reviewed in Ref. 3). The suggestion that opioids and opiates

[a] mCPP was classified as a 5-$HT_{1C/1D}$ agonist when these studies were conducted. More recently the 5-HT_{1C} receptor has been renamed the 5-HT_{2C} receptor in Ref. 709. We have changed the originally published nomenclature to be consistent with the current classification.

generally inhibit sexual behavior is based in part on the facilitative effects of antagonists, especially in sexually sluggish males,[225] sexually satiated males,[176] or sexually naive males tested in a novel environment.[112] Male quail also showed facilitation of copulatory behaviors, but not anticipatory behaviors, by centrally administered naloxone.[226] However, systemic administration of the opiate antagonists naloxone and naltrexone have also had inhibitory effects, including increased postejaculatory intervals[227,228] and decreased ability of mild tail pinch to stimulate copulation in castrates with subnormal T replacement.[229]

A second line of evidence for inhibitory effects of opiates is that morphine or the agonists U-50,488H or bremazocine inhibited sexual behaviors in active copulators.[230,231] However, inhibitory effects of the opioid antagonist naloxone on active copulators[230] suggests that at least a minimal amount of activity at opioid receptors can facilitate copulation, but that an excess may impair sexual behavior. Some inhibitory effects of systemically administered morphine may result from inhibition of genital reflexes. All doses of morphine suppressed seminal emissions and touch-based erections.[232] However, naloxone also inhibited touch-based erections in those experiments, again suggesting that some opioid activity can facilitate sexual function. Furthermore, naloxone also inhibited anticipatory level-changing in a bilevel apparatus,[228,233] suggesting that some endogenous opioid activity facilitates sexual motivation. Opioid facilitation may result from disinhibition of the mesolimbic DA system, as there are receptors on inhibitory GABAergic neurons in the VTA. Increasing levels of enkephalins, by intraventricular administration of an enkephalinase inhibitor, decreased both the time and number of intromissions preceding ejaculation, but also increased mount and intromission latencies.[234] Thus, low levels of opioid activity may facilitate genital reflexes and sexual motivation; however, excess activity can interfere with sexual behavior, and some measures of behavior may be more sensitive than others to such interference. Enkephalins were increased in tissue from several brain areas following copulation either to one ejaculation or to exhaustion, with no significant difference between the two groups.[234a] These authors also reported that sexually inactive males had generally higher enkephalin content than did sexually active males, again supporting an inhibitory effect of relatively high opioid levels.

Oxytocin

Oxytocin is a nonapeptide that is synthesized in the paraventricular (PVN) and supraoptic nuclei of the hypothalamus and released both from terminals in the posterior pituitary and into the CNS. Circulating oxytocin from the PVN and posterior pituitary stimulates smooth muscles, thereby facilitating seminal emission and, in humans, contributing to orgasm. In female mammals it stimulates milk let-down and parturition (see Chapter 13). Plasma oxytocin was increased by copulation in naive, but not sexually experienced, male rats.[235] In the naive males, plasma oxytocin was correlated with intensity of copulation. Systemically administered oxytocin also restored copulation in males whose copulatory behavior had been impaired by chronic fluoxetine.[189] Facilitative effects of low doses of oxytocin have been observed in rats[236] and dominant squirrel monkeys,[237] but high doses have inhibited copulation in prairie voles[238] and rats.[239] A facilitative effect of endogenous oxytocin on copulation was supported by the dose-related inhibitory effects of an oxytocin antagonist, administered ICV.[240]

Intraventricular injections of oxytocin also increased spontaneous erections, and this increase was blocked by an oxytocin antagonist or an anticholinergic drug (atropine), but not by a DA antagonist.[241] The oxytocin antagonist also blocked erections elicited by the DA agonist apomorphine,[241] suggesting that DA is "upstream" of oxytocin in the control of drug-induced erections; ACh may, in turn, be "downstream" from oxytocin. Finally, an oxytocin antagonist, administered ICV, inhibited NCE, suggesting a role for oxytocin in the control of erections elicited by normal physiological stimuli, as well as drug-induced erections (reviewed in Ref. 242).

Nitric Oxide

NO is a soluble gas produced in the conversion of L-arginine to citrulline by NOS. NO contributes both peripherally and centrally to vasodilation, erection, and other parasympathetic functions. Both neuronal (nNOS) and endothelial (eNOS) isoforms of NOS are present in the corpus cavernosum and dorsal penile nerve and are upregulated by T or DHT.[243–247] Castration decreased, and exogenous T restored, erectile responses elicited by cavernous nerve stimulation, systemically administered apomorphine, and/or injection of the nonspecific phosphodiesterase inhibitor papaverine into the penis (reviewed in Ref. 248). Phosphodiesterases inactivate cGMP, the most common second messenger of NO, as well as cAMP; therefore, their inhibition would prolong NO's effects. Indeed, sildenafil, vardenafil, tadalafil, and other drugs used to treat erectile dysfunction inhibit PDE5, which is more selective for cGMP (reviewed in Ref. 3). Sildenafil did not affect erections in healthy men but did decrease the interval between ejaculation and a subsequent erection.[249] Therefore, sildenafil can increase penile vasodilation in men with erectile dysfunction and can facilitate postejaculatory erections in healthy men, but not pre-ejaculatory erections. In rats, too, sildenafil increased the occurrence of

penile erections, as well as genital grooming and homosexual mounting,[161] and vardenafil increased intracavernous pressure in anesthetized rats to a greater extent than did sildenafil.[250]

Although castration decreased NOS in the penis and epididymis, it actually increased NOS in the seminal vesicles and lateral prostate; exogenous T in castrates decreased NOS to normal levels in the latter structures.[251] Furthermore, systemic administration of L-arginine impaired fertility in male rats, without inhibiting sexual behavior.[252] Thus, excessive NO in some parts of the reproductive tract may impair aspects of ejaculation or sperm motility.

Systemically administered NOS inhibitors have decreased the numbers of sexually experienced[253,254] and inexperienced[255] males that could ejaculate, increased their mounts, and decreased their intromissions. NOS inhibitors have also decreased the number of NCE[256] and touch-based erections,[254] but increased the number of seminal emissions,[254] consistent with a facilitative role for NO on parasympathetic function and inhibition of sympathetic activity. Sexual motivation, measured as percent choice of a female in an X-maze[254] or as increased mount latency,[253] was not affected by NOS inhibitors, although another study reported a decrease in motivation, measured as decreased precoital activity and decreased numbers of animals that mounted.[257] In the latter study, the numbers of spermatozoa were normal, but the number of pregnancies and the number of implanted embryos per pregnancy were lower than in controls. One explanation for the impaired fertility may be the requirement for NO to be injected by the sperm into the egg to elicit release of internal stores of calcium, which in turn triggers cell division.[258] In nNOS "knockout" mice, normal penile function was found, as a result of increased production of eNOS.[259] Indeed, nNOS−/− mice required fewer mounts and intromissions to ejaculate,[260] again suggestive of NO's normal role in activating parasympathetic and inhibiting sympathetic function. Thus, NO may help to prevent premature ejaculation.

There have been inconsistent results of systemically administered NO donors or precursor. L-arginine increased the number of naive male rats that copulated and improved the performance of sexually experienced males.[255] Production of NO in the corpus cavernosum was also increased by L-arginine.[54] However, the NO donor sodium nitroprusside increased intracavernosal pressure only in untreated castrates, not in T-replaced males.[246] Also, there were only marginal facilitative effects of L-arginine on touch-based erections,[254] and no effects on intracavernosal pressure elicited by electrical stimulation in castrates, with or without T replacement.[246] Therefore, NOS activity in many gonadally intact males may be sufficient for normal copulation and may not benefit from excess precursor or NO donors.

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH), a decapeptide, is produced by neurons in the mediobasal hypothalamus, the lamina terminalis, and the MPOA.[261] After release in the median eminence, GnRH is carried into the hypophyseal portal system to the anterior pituitary, where it stimulates gonadotropin release. It also acts as a neuromodulator in several brain areas, including the MPOA and amygdala.[261,262] It is released in response to female pheromones in mice and hamsters (reviewed in Ref. 263).

Administration of GnRH has resulted in inconsistent effects. Moss and his colleagues reported that GnRH, administered to castrated male rats maintained on a low dose of T, decreased their intromission and ejaculation latencies; gonadally intact males were not affected.[264] Myers and Baum,[265] however, reported facilitative effects only in intact males, not in T-replaced castrates; indeed, GnRH lengthened the postejaculatory interval (PEI) of quiescence in T-treated castrates. Lengthened PEI was also observed in old rhesus monkeys.[266] GnRH had no effects in male rats in another study,[267] but increased the mount rate in male rats with anesthetized genitalia in another experiment[268] and decreased intromission and ejaculation latencies in gray-tailed voles.[269] GnRH administered ICV also promoted the mating behavior of sexually naive male hamsters whose vomeronasal organs had been removed (VNX).[270] Previous sexual experience is usually necessary for VNX male hamsters to copulate normally; thus, administration of GnRH compensated for the lack of sexual experience. Similarly administered GnRH increased Fos immunoreactivity (IR) in the MPOA of male hamsters in response to female pheromones; it also increased the MPOA Fos response in sexually experienced VNX hamsters.[271] Sexually naive VNX males showed enhanced Fos-IR in the medial amygdala (Me), but not the MPOA, in response to pheromone plus ICV GnRH. In hypogonadal men with decreased LH release, GnRH improved sexual function.[272] The authors suggested that the improvement was not due to increased androgen levels, because free T was well below the normal adult range when the improvement in function began. However, GnRH resulted in little[273,274] or no[275] improvement in other men with erectile dysfunction.

Chronic administration of a synthetic GnRH agonist ([6-*D*-(2-napthyl)-alanine]GnRH) disrupted copulation in gonadally intact male rats and also decreased levels of T.[276] The likely explanation for this inhibitory effect is that endogenous GnRH is released in a pulsatile fashion, and continuous high doses of GnRH inhibit LH release and gonadal function.[277,278] Thus, GnRH agonists have

been suggested as antifertility drugs.[279] Apparently, they also inhibit sexual behavior, perhaps as a result of the decrease in T.

Alpha-Melanocyte Stimulating Hormone and Related Peptides

Alpha-melanocyte stimulating hormone (α-MSH) is secreted into the systemic circulation from the intermediate lobe of the pituitary; it and related melanocortin peptides, derived from the precursor proopiomelanocortin (POMC), are also expressed in numerous CNS and peripheral structures. Besides α-MSH, these peptides include adrenocorticotropic hormone (ACTH) and β-lipotropin, released from the anterior pituitary; the opioid endorphin; and other variants formed by posttranslational processing. They bind to a family of five G-protein-coupled receptors (MC-1 to MC-5), all of which activate adenylyl cyclase (see Ref. 280 for an excellent review).

Alpha-MSH and ACTH, administered ICV, have been shown to increase erections in dogs, cats, rats, mice, and rabbits as early as the 1960s (reviewed in Ref. 280). Other commonly reported effects have been decreases in the numbers of mounts and intromissions preceding ejaculation and in latency to ejaculate. Both α-MSH and ACTH elicited penile erections, and stretching and yawning in rats, when injected into the third ventricle; the stretching and yawning were blocked by an MC-4 receptor antagonist, but penile erection was not,[281] suggesting that a different MC receptor was important for facilitation of erection (but see below). However, ACTH-induced erections were inhibited by either ICV or systemic injections of the NOS anatagonist L-NAME, suggesting that the effects of ICV ACTH were mediated by increased central production of NO.[282]

There has been a resurgence of interest in these peptides with the production of several synthetic peptides that are selective for subtypes of melanocortin (MC) receptors. Two of these have been reported to increase erections and sexual interest in men (melanotan-II (MT-II)[283–286] and PT-141[287]), with nausea, stretching, yawning, and decreased appetite reported as side effects of MT-II. MT-II is a selective melanocortin-4 (MC-4) agonist that has also increased erections in rats, especially after intrathecal injections, although ICV and IV injections were also effective.[288] The MC-3/MC-4 receptor antagonist SHU 9119 blocked the effects of MT-II, confirming its receptor specificity. Intracavernosal injections had no effect, suggesting that MC receptors in the spinal cord and brain mediate the pro-erectile activity. In rabbits, too, IV injections of MT-II increased cavernosal pressure, and again isolated cavernosal tissue was unresponsive.[289] Both SHU 9119 and the NOS inhibitor L-NAME abolished the effects of MT-II, indicating that stimulation of central MC-3/MC-4 receptors achieves the proerectile effects by releasing NO. L-NAME also blocked the proerectile effects of ICV administration of ACTH[282] and of α-MSH and oxytocin[290] in rats. Furthermore, MT-II enhanced copulatory behavior and augmented erections elicited by cavernous nerve stimulation in wild-type, but not MC-4 receptor knockout, mice.[284] In addition, MC-4 receptors were expressed in nerve fibers and mechanoreceptors in both rat and human penis, but not in cavernosal smooth muscle; expression was also observed in rat spinal cord, brainstem, hypothalamus, and pelvic gangion.[284] Intranasal administration of the MC-4 agonist PT-141 also elicited drug-induced erections in rats, as well as in men with erectile dysfunction.[287] Finally, both systemic and ICV administration of a different, highly selective MC-4 receptor agonist, THIQ, increased intracavernous pressure and elicited reflexive erections in rats; these effects were blocked by both the endogenous nonselective antagonist, agouti-related protein, and a synthetic MC-4-preferring antagonist, MBP10.[291] These effects were also attenuated by an oxytocin antagonist.

In summary, endogenous α-MSH and ACTH, as well as synthetic MC-3 and MC-4 receptor agonists, may promote erection by acting synergistically at nerve endings in the penis, in the lower spinal cord, and in several areas of the brain. Some of these effects are mediated by NO, and perhaps also by oxytocin.

Injection of prostaglandin E_2 (PGE_2) into the fourth ventricle of male rats decreased the postejaculatory interval (see Ref. 292; reviewed in Ref. 293). This treatment also increased basal forebrain temperature, measured in the MPOA. As noted above, temperature in this area increases during copulation and decreases during the postejaculatory interval. It is not clear whether PGE_2 influences copulation directly, or through the increase in brain temperature. It is interesting that manipulations of PGE_2 during early development influenced masculinization of rats.[294]

Other Peptides

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) contributes to penile erection through stimulation of adenylyl cyclase in the corpus cavernosum, thereby relaxing smooth muscle (reviewed in Ref. 52). Antagonists to VIP and ACh additively reduced erection elicited by pelvic nerve stimulation; however, the additive facilitative effects of VIP and ACh did not elicit a full erectile response.[295] Thus, while both VIP and ACh contribute to erection, they do not appear to be principal neurotransmitters for that effect. VIP may facilitate copulation, apart from its effects on

erection. Systemic injection of VIP to castrated male rats, maintained on suboptimal T replacement, reduced intromission latency and interintromission interval; these effects were blocked by a VIP antagonist administered with VIP.[296]

Galanin and Galanin-Like Peptide

Better known for its role in stimulating feeding, galanin, microinjected ICV, decreased mounts and intromissions in male rats.[297] A galanin antagonist (galantide) increased those behaviors.[298] In contrast to these apparently inhibitory effects of galanin, microinjection of the peptide into the MPOA of castrated, T-replaced rats facilitated their copulatory behavior,[299] and ICV injections of a galanin antagonist in ferrets decreased their time spent near a receptive female, although the injections did not affect copulation.[300] It is not clear whether these contradictory findings reflect site, species, dosage, or other differences. Galanin-like peptide (GALP) shares partial sequence homology with galanin but is derived from a different gene. ICV administration of GALP facilitated several measures of male sexual behavior in male rats, whereas similar administration of galanin inhibited those same measures.[301] GALP is known to stimulate GnRH secretion; however, GALP increased sexual behavior even in castrated males, suggesting that effects on testosterone secretion did not mediate the behavioral effects.

Neuropeptide Y

Neuropeptide Y (NPY) is widely distributed throughout the brain and is often co-localized with NE.[302] NPY, administered ICV, stimulated feeding, but inhibited copulation in male rats.[303,304] Consistent with an inhibitory effect of NPY, ICV administration of an NPY antagonist facilitated copulation.[305] However, systemically administered NPY failed to affect reflexive erections.[303]

Prolactin

A large number of studies in the 1980s revealed inhibitory effects of chronic prolactinemia, induced by implanting excess pituitary glands or prolactin-secreting tumors, by injecting drugs that stimulate PRL release, or by direct injection of PRL (reviewed in Ref. 3). The most commonly reported effects were increased mount and ejaculation latencies and general slowing of copulation. However, there were some contradictory effects, and the mechanism by which chronically elevated prolactin may impair sexual behavior is not clear. A review of hormonally induced dysfunction in men found that severe hyperprolactinemia, often caused by a pituitary tumor, resulted in low T, erectile dysfunction, and decreased desire, likely due to suppression of gonadotropin secretion.[306]

Corticotropin-Releasing Hormone

Injections of corticotropin-releasing hormone (CRH) into the third ventricle increased mount, intromission, and ejaculation latencies and increased the numbers of mounts and intromissions preceding ejaculation.[307] These inhibitory effects of CRH were attenuated by co-administration of the opioid antagonist naloxone. Furthermore, CRH can inhibit GnRH release, possibly acting through endogenous opioids.[308] The promotion of freezing and flight behaviors by CRH, in addition to its inhibition of sexual behavior, suggests that it organizes a constellation of behaviors appropriate to stressful or threatening situations.

Cholecystokinin

There have been mixed reports of cholecystokinin (CCK) effects on male sexual behavior. One study found that systemic administration of sulfated CCK decreased the numbers of intromissions and the time preceding ejaculation; these effects were reversed by the CCK antagonist proglumide.[309] However, other studies found no effect of systemic[310,311] or ICV[310,312] administration of CCK. On the other hand, there are at least two subtypes of CCK receptor,[313] which may have opposite effects. Thus, the failure to find significant effects of CCK may be due to opposing excitatory and inhibitory effects (see the section Mesocorticolimbic DA Tract later in the chapter).

Angiotensin II

Angiotensin II, microinjected into the third ventricle, increased intromission latency, the number of intromissions preceding ejaculation, and postejaculatory interval.[314] The most effective dose also increased drinking during the test. A more recent study confirmed the inhibitory effects, which were blocked by an angiotensin I receptor antagonist administered in the drinking water.[315] However, it is not clear whether the sexual impairment was a direct effect or a result of thirst.

BRAIN AREAS IMPLICATED IN CONTROL OF MALE SEXUAL BEHAVIOR

Sensory Systems

Olfactory Bulbs

Chemosensory cues contribute to male sexual behavior across a wide variety of species, from rodents to primates. However, the relative importance of chemosensory stimuli, compared with other sensory cues, differs widely among species. Chemosensory cues are especially important for mating in rodents and other nocturnal species that mate in underground burrows.

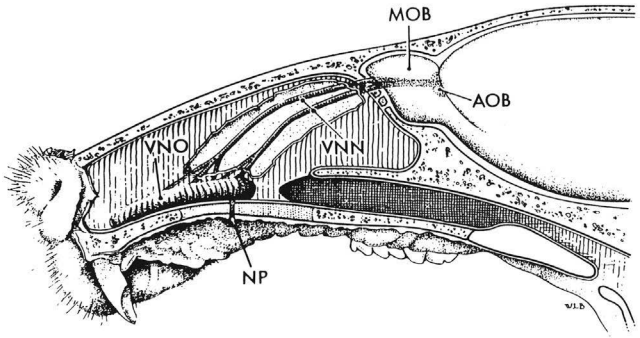

FIGURE 49.8 Relation between the vomeronasal organ and the olfactory bulbs of the Syrian hamster. The vomeronasal organ (VNO) projects to the accessory olfactory bulb (AOB) via the vomeronasal nerve (VNN). The vomeronasal nerve travels medial to the main olfactory bulb on the way to the accessory olfactory bulb. Olfactory information is presumed to reach the vomeronasal organ through the nasopalatine duct. In contrast, the main olfactory bulb receives innervation from the olfactory nerve originating from receptors in the nasal mucosa (pathway not shown). AOB, accessory olfactory bulb; MOB, main olfactory bulb; NP, nasopalatine duct; VNN, vomeronasal nerve; VNO, vomeronasal organ. *Source: We appreciate the generosity of Sarah W. Newman for providing this illustration adapted from one previously published in Ref. 316.*

The olfactory system includes two functionally and anatomically distinct components (see Figure 49.8). The main olfactory bulbs (MOBs) receive information about volatile odors from receptors in the nasal epithelium, which project through the cribriform plate to the MOBs. The accessory olfactory bulbs (AOBs) receive both volatile and nonvolatile species-specific information from receptors in the vomeronasal organ (VNO), which consists of bilateral tunnels along the base of the nasal cavity.[317] The main and accessory olfactory bulbs have parallel but overlapping projections to amygdaloid nuclei through the lateral olfactory tract; bilateral transection of that tract blocked mating, presumably by interrupting olfactory bulb efferents to the amygdala.[318]

Stimuli transduced in the VNO are especially important for social behavior, including mating, aggression, affiliation, and maternal behavior. However, in species with relatively poorly developed olfactory systems, including humans, the VNO and AOB are greatly diminished, and some have argued that they may be nonfunctional (reviewed in Ref. 319).

EFFECTS OF LESIONS

Damage to the olfactory system impairs male sexual behavior of numerous species (reviewed in Ref. 3). In hamsters, bilateral bulbectomies abolished sexual behavior and the mating-induced increase in extracellular MPOA dopamine, which was seen in hamsters with sham lesions and also contralateral to unilateral lesions.[320] Sexually naive hamsters showed a more pronounced reduction in sexual behavior after vomeronasal organ removal, compared to experienced males.[321] Destruction of receptors in the nasal epithelium of hamsters, together with vomeronasal nerve cuts or deafferentation of the vomeronasal pump, which normally sucks mucus with dissolved chemosensory stimuli into the VNO, also severely disrupted copulation.[322] There was minimal disruption of sexual activity after deafferentation of the main olfactory bulb by irrigation of the nasal cavity with zinc sulfate to destroy olfactory receptor neurons (reviewed in Ref. 3). However, combined deafferentation of the main and accessory olfactory systems eliminated mating, similar to the effects of bilateral olfactory bulbectomy.[316]

In rats, olfactory bulbectomy reduced the percentage of males that copulated to ejaculation and delayed the onset of copulation in those males that did mate (reviewed in Ref. 3). However, preference for a receptive female over a nonreceptive female was unaffected.[323] In rats, olfactory bulbectomy also eliminated NCE elicited by female rat urine.[16] As in male hamsters, sexually naive male rats may be more sensitive to anosmia,[324] although severe copulatory dysfunction has also been reported following olfactory bulbectomy in sexually experienced males.[325,326] However, increasing general arousal could activate copulation in male rats after olfactory bulbectomy. Mildly painful flank shock or tail pinch stimulated copulation in olfactory bulbectomized male rats, but these anosmic animals still exhibited longer intromission and ejaculation latencies compared with controls, and the effects of stimulation were transient.[325,326] Interestingly, NCE appear to be stimulated by the main olfactory system, since lesions of the vomeronasal organ had no effect, but zinc sulfate lesions of the olfactory epithelium significantly decreased NCE.[16] Also, deafferentation of both the MOB and AOB severely decreased NCE and inhibited mating.[327]

Chemosensory cues are also important for mating in male mice. Olfactory bulbectomy eliminated mounting in essentially all male mice, although zinc sulfate destruction of receptors in the olfactory epithelium did not interrupt mating (reviewed in Ref. 3). Although early studies reported that VNO lesions sharply decreased the proportion of male mice that intromitted and ejaculated,[328] recent studies have found no significant reduction in mating or in the ability to discriminate estrous from nonestrous urine.[329] Male mice with null mutations of the transient receptor potential 2 (TRP2) cation channel to disrupt VNO receptors showed high levels of mounting of male intruders as well as normal sexual behavior with females.[330] However, bilateral lesions of the VNO or AOB did decrease the amount of time that male mice spent investigating the urinary volatiles from estrous females.[329,331] Based on these recent results, it appears that the vomeronasal system in mice has an important,

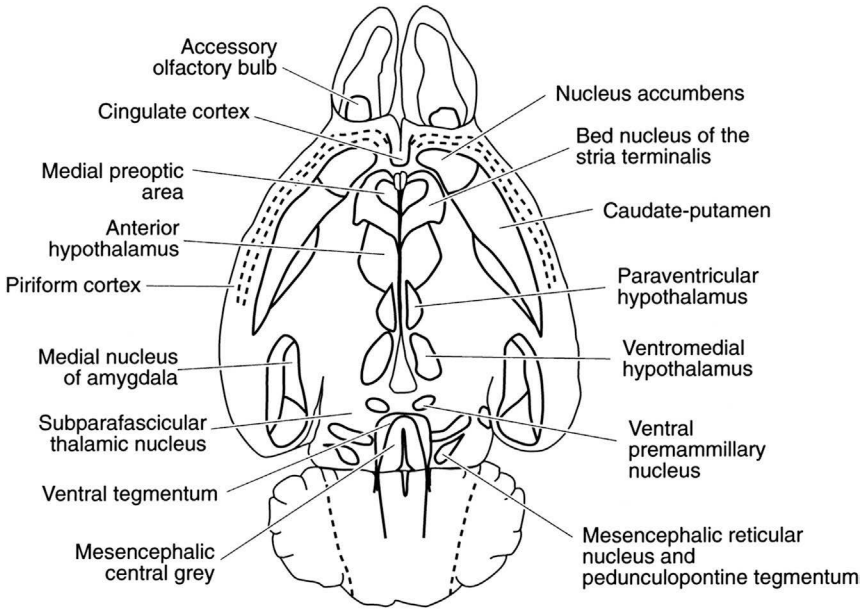

FIGURE 49.9 Regions within a horizontal section of rat brain in which Fos induction is observed after sexual stimulation in females and males. With some notable exceptions, Fos has been observed in similar regions in hamsters and gerbils. *Source: Figure is reprinted from Ref. 332, with permission.*

but not critical, role in copulation. Destruction of the main olfactory receptors by zinc sulfate did significantly impair detection of the volatile estrous odors.[331] Thus, only the main olfactory system is necessary for male mice to detect estrous odors at a distance, but the VNO system contributes to detection in close proximity and to preference for estrous odors.

The role of the olfactory system in male sexual behavior in other species has received less attention. Olfactory bulbectomy produced variable deficits in the behavior of male guinea pigs, and failed to block copulation in male dogs, cats, rhesus monkeys, gerbils, sheep, or ferrets, although naris occlusion blocked heterosexual partner preference in ferrets (reviewed in Ref. 3). In early studies, the effects of olfactory bulbectomy were attributed to the resulting anosmia. However, the olfactory bulbs receive ascending inputs from olfactory-related structures and from nonolfactory subcortical areas, so both olfactory and nonsensory functions of the olfactory bulbs may contribute to reproductive and social behaviors.

ACTIVATION OF *C-FOS* OR OTHER IMMEDIATE–EARLY GENES

Although lesions provide information about brain areas that are critical for behavior, they may fail to identify other areas that contribute to behavioral performance. Activation of immediate-early genes provides a complementary approach to map brain circuits that are activated during behavior (reviewed in Ref. 332; see Figure 49.9). Copulation by male hamsters increased Fos-IR in the AOB and their central projection sites; this increase was also observed in males whose main olfactory system was ablated with zinc sulfate.[333] (See Figure 49.9 for a diagram of the brain areas activated by sexual behavior in male rodents.) Similarly, Fos expression in efferent targets of the MOB was maintained after vomeronasal lesions in sexually experienced male hamsters,[334,335] but in sexually naive males with vomeronasal lesions, odors alone were not able to stimulate Fos-IR.[335] Therefore, pheromonal stimulation of the vomeronasal organ is sufficient for Fos induction in the accessory olfactory bulb and its central targets, but odors transmitted through the main olfactory system may also induce Fos-IR, particularly in males with prior sexual experience. The increase in Fos-IR in the olfactory bulbs of male hamsters was specific to chemosensory stimuli rather than mating, because female odors alone induced Fos-IR (reviewed in Ref. 3). Although T is required for sexual activity, Fos expression in the olfactory bulb was unaffected by castration,[336] suggesting that castration may alter downstream responses to sex-related chemosensory cues, but not the detection of those cues.

As in hamsters, mating or exposure to estrous female bedding stimulates Fos in the AOB and downstream structures in male rats and mice.[337–339] Mating elicits more Fos than does chemosensory stimulation alone[340]; however, NCE in male rats fail to enhance Fos expression.[341] Within the accessory olfactory bulb in male mice, Fos induction by female stimuli is concentrated in the rostral region, and is blocked by removal of the vomeronasal organ.[342,343] This supports the existence of functional heterogeneity in the accessory olfactory system.

Although the accessory olfactory system appears to be more important than the main olfactory system for Fos induction in rodents, male ferrets, which are carnivores,

rely on the main olfactory system.[344] Both estrous female bedding and peppermint odor increased Fos in the main olfactory bulb of castrated male ferrets. However, female pheromones failed to stimulate Fos in the accessory olfactory bulb. In male ferrets, unlike male hamsters, T treatment increased the Fos response to female odors, but not to peppermint. Moreover, the ferret olfactory bulb has ARs. Thus, in this species, T has the potential to increase the responsiveness of olfactory bulb neurons to sex-related chemosensory stimuli.

Somatosensory Systems

Mechanoreceptors in the penis provide somatosensory information to the brain and stimulate sexual arousal. They are more responsive when the penis is erect[345] or near core body temperature, as it is during erection.[346] Somatosensory input from the penis enters the spinal cord mainly via the dorsal nerve of the penis (reviewed in Ref. 347). Ejaculation-specific information from the lumbar spinal cord is relayed to the thalamus; targeted toxin lesions of these cells completely disrupted ejaculation in rats.[43]

Auditory System

Male and female rats produce ultrasonic vocalizations before and during copulation; these vocalizations are believed to facilitate copulation (reviewed in Ref. 348). During mating, both male and female rats produce 50-kHz vocalizations, which are associated with arousal, and males produce a 22-kHz vocalization following ejaculation.[349] Vocalizations are influenced by hormones, as Silastic capsule implants with a high concentration of testosterone reinstated mating-induced vocalization in castrates, but blank implants did not.[90]

Amygdala

There is some controversy as to whether the amygdala should be considered an entity at all, rather than a conglomeration of dissimilar areas related to learning, motivation, and fear (central nucleus and basolateral division) or to chemosensory stimuli and social behavior (corticomedial division) (see Figure 49.10).[351] In rodents, the corticomedial amygdala is a key nodal point for integration of chemosensory, somatosensory, and hormonal stimuli. In particular, the Me relays chemosensory stimuli from the olfactory bulbs to midline nuclei of the preoptic area and hypothalamus.

During mating, the amygdala receives chemosensory, somatosensory, and hormonal stimuli via the main and accessory olfactory bulbs, the thalamic subparafascicular nucleus, and steroid receptors, respectively. Chemosensory information is then transmitted by the projections of mitral and tufted cells in the olfactory bulbs through the lateral olfactory tract, terminating in the medial and cortical amygdaloid nuclei. Efferent projections of the accessory olfactory bulb target the medial and posteromedial nuclei, while the main olfactory bulbs project widely to the anterior and posterolateral cortical nuclei and to olfactory processing areas in ventral allocortex (see Figure 49.11). In addition, the Me receives ascending somatosensory cues from the penis and perineal region relayed through the subparafascicular nucleus. However, projections of the subparafascicular nucleus are not limited to the amygdala; somatosensory cues are conveyed directly to the MPOA, as well.

The medial and posteromedial cortical nuclei have abundant receptors for androgens and estrogens. T promotes male sexual activity, at least in part, through

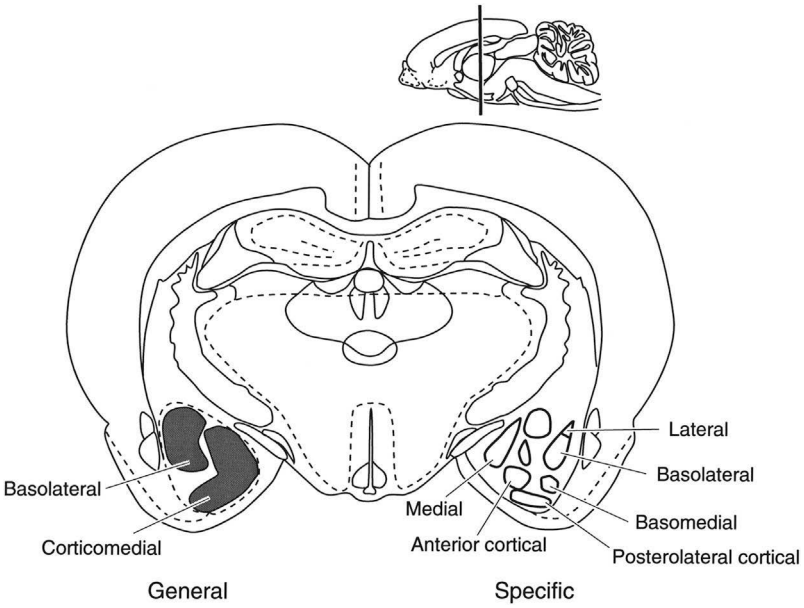

FIGURE 49.10 The amygdala, an almond-shaped structure located in each temporal lobe, seen in a schematic drawing of a coronal section of the rat brain. Two general amygdaloid regions, the basolateral and corticomedial nuclei (left), have been studied extensively. Destruction of the corticomedial nuclei, but not the basolateral nuclei, severely affects male copulatory behavior in rodents. The specific nuclei of the basolateral and corticomedial amygdala are depicted (right). *Figure is reprinted from Ref. 350, with permission.*

binding to amygdaloid ARs or to ERs after local aromatization. However, steroid receptors are also concentrated in midline nuclei of the preoptic area and hypothalamus, including the medial preoptic area (MPOA). Within the Me, ARs and ERs are localized preferentially in the posterior subnucleus, whereas olfactory bulb efferents terminate preferentially in the anterior subnucleus.

Effects of Lesions

The impact of amygdala lesions on male sexual behavior depends on the specific sites within the amygdala. In general, lesions of the central nucleus or the basolateral division (basolateral, basomedial, and lateral nuclei) do not disrupt copulation in male rats or hamsters, although there is some indication that male rats copulate more rapidly following basolateral amygdaloid lesions (reviewed in Ref. 3). The central and basolateral regions of the amygdala have connections with the frontal cortex, brainstem areas for autonomic control, and ventral striatum (see Ref. 351), and are important for conditioned responses during fear and reinforcement. Everitt and co-workers showed that basolateral amygdala lesions abolished an operant task (bar-pressing) for a secondary reinforcer that had been previously paired with access to a receptive female; however, those lesions did not affect copulation (reviewed in Ref. 353). Therefore, the basolateral amygdala may be important for motivational aspects of mating, and for learning appropriate associations, but not for copulatory performance. In support of this conclusion, exposure to sexually conditioned almond odor increased activation of the basolateral amygdala, while exposure to soiled bedding from an estrous female did not.[354]

In contrast, corticomedial amygdala lesions do impair copulation in rats,[355,356] hamsters,[357] and gerbils.[358] These lesions reduced copulatory efficiency, increased mount and ejaculation latencies, increased intromissions preceding ejaculation, increased inter-intromission intervals, and decreased the number of ejaculations before sexual exhaustion. In addition, Me lesions blocked the facilitative effects on copulation of pre-exposure to an estrous female[359] and reduced the number of NCE.[199] Thus, the Me facilitates the response to and assimilation of sexually exciting stimuli.

Through its efferent projections to the MPOA, the Me facilitates appetitive sexual behavior and subsequent

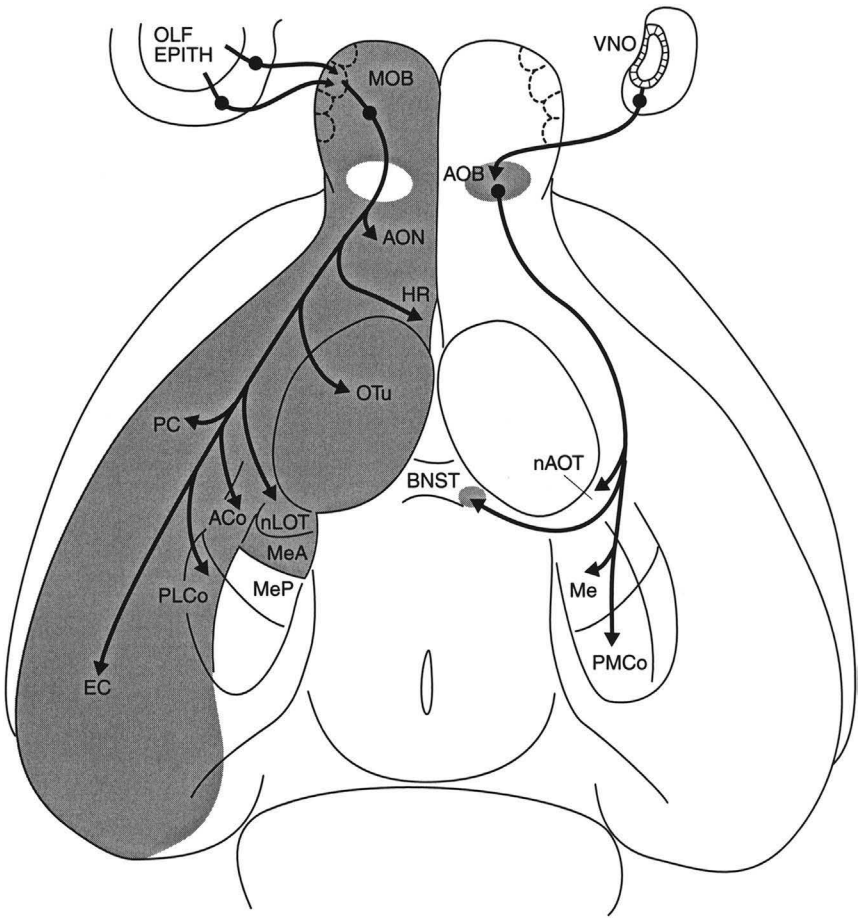

FIGURE 49.11 Diagram of the ventral surface of the hamster brain. Shaded areas indicate brain regions that receive efferent projections of the olfactory system, via the main olfactory bulb (left), and of the vomeronasal system, via the accessory olfactory bulb (right). ACo, anterior cortical nucleus of the amygdala; AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; BNST, bed nucleus of the stria terminalis; EC, entorhinal cortex; HR, hippocampal rudiment; Me, medial nucleus of the amygdala; MeA, medial nucleus of the amygdala, anterior division; MeP, medial nucleus of the amygdala, posterior division; MOB, main olfactory bulb; nAOT, nucleus of the accessory olfactory tract; nLOT, nucleus of the lateral olfactory tract; OLF EPITH, olfactory epithelium; OTu, olfactory tubercle; PC, piriform cortex; PLCo, posterolateral cortical nucleus of the amygdala; PMCo, posteromedial cortical nucleus of the amygdala; VNO, vomeronasal organ. *Source: Figure is reprinted from Ref. 352, with permission.*

copulation. Unilateral lesions of the MPOA impaired, but did not eliminate mating in male rats.[360] However, when combined with lesion of the contralateral Me, copulatory behavior was severely disrupted. Similar effects have been reported in gerbils.[358] Likewise, chemical stimulation of the Me increased DA release in the MPOA,[361] and microinjection of the DA agonist apomorphine into the MPOA restored sexual behavior inhibited by Me lesions.[355] As measured by microdialysis, basal DA levels in MPOA were unaffected in males with Me lesions, but there was no DA release in response to a receptive female or during copulation.[355] These observations suggest that the Me is critical for copulatory behavior organized through the MPOA. Because there are no DA-containing neurons in the Me of rats, amygdala efferents apparently synapse, either directly or indirectly, on dopaminergic cell bodies or terminals in the MPOA. Using a somewhat different model for sexual motivation, lesions of the posterior Me abolished NCE and reduced preference for an estrous female in a partner preference test, but had no effect on copulatory behavior.[362] Finally, a subregion of the posterodorsal Me appears to be associated with sexual satiety. Lesions of this area in male hamsters increased the number of ejaculations preceding satiety, and Fos expression correlated with the onset of satiety.[363]

Activation of c-fos *or Other Immediate–Early Genes*

The olfactory system projects to the medial and anterior cortical nuclei of the amygdala (reviewed in Ref. 364). Both chemosensory stimuli from estrous female rats and increasing amounts of copulation elicited increasing amounts of Fos-IR in the Me and in several "downstream" sites that are important for male sexual behavior.[337,339,365] Chemosensory stimuli and copulation also induced increasing Fos-IR patterns in hamsters, gerbils, prairie voles, and musk shrews (reviewed in Ref. 3). Information about opposite-sex odors in hamsters is processed in the anterior Me and is then relayed to the posterodorsal Me (MePD), which generates attraction for those odors.[366] Disconnection of these subregions eliminated male hamsters' preference for opposite-sex odor.[367]

Anesthetizing the penis of male rats with lidocaine resulted in few intromissions, but a significant increase in Fos-IR in both the Me and MPOA,[368] suggesting that chemosensory stimuli are the primary determinants of the mounting-induced increments in neuronal Fos expression. Likewise, exposure of male rats to bedding from estrous females increased Fos-IR at each level of the vomeronasal circuit, including the Me.[369] In hamsters, exposing males to a cotton swab containing female hamster vaginal secretions elicited similar results.[333,370,371]

Chemosensory cues for Fos induction in the Me can be further subdivided into those transduced by the main and accessory olfactory systems. Intranasal zinc sulfate treatment to damage the olfactory mucosa without affecting the vomeronasal system decreased copulation-induced Fos-IR in the anterior Me of male hamsters,[372] suggesting that input from the main olfactory system contributes to the activation of neurons in this area. In sexually experienced male hamsters, vomeronasal lesions had minimal effect on Fos expression in response to female stimuli, but similar lesions practically eliminated this Fos in the Me in naive males.[335]

The MePD contains a high concentration of ARs. Some of those androgen-sensitive neurons in male rats project to the MPOA and are activated selectively by ejaculation.[373] In male gerbils, too, the lateral part of the MePD was activated only following ejaculation, whereas the medial part was activated by sex-related odors[374] (see the section Circitry and Anatomical Interconnections later in the chapter). Many of those mating-activated (Fos-IR) cells in the lateral MePD of gerbils, as well as in two preoptic nuclei (posterodorsal and medial preoptic nuclei), contained NOS, and many of those NOS neurons also expressed Fos or AR.[375] The authors suggested that NO may help synchronize neural activity in those areas to either trigger ejaculation or respond to ejaculation. The MePD is also part of an interconnected ejaculation-specific circuit that may promote sexual satiety in rats, hamsters, and gerbils (reviewed in Ref. 3). Male rats had a higher regional and neuronal optical density of NADPH-diaphorase (NADPH-d), a marker of NOS, in the MePD; however, there was no sex difference in numbers of cells that express NADPH-d.[376] Thus, there may be similar numbers of NADPH-d neurons in males and females, but there is more NADPH-d, and presumably NOS, in each neuron in males.

The androgen-sensitive cells may also be important for sexual arousal, as the androgen receptor antagonist flutamide in the medial amygdala decreased the number of, and increased the latency to, "psychogenic" NCE.[377] Furthermore, activation of "downstream" forebrain areas following glutamatergic stimulation of the medial amygdala is steroid dependent.[378]

Mating did not induce significant Fos expression in the central and basolateral nuclei. Interestingly, the distribution of mating-induced Fos within the Me does not precisely accord with the results of tract tracing and lesion studies. Although the anterior subdivision receives the majority of direct olfactory bulb efferents, and lesions of this area produce greater deficits in copulatory behavior,[357] the largest concentration of Fos-positive neurons is found in the posterior subnucleus of the Me.[379] Furthermore, activation of the posterodorsal subdivision of the Me (MePD) appears to be associated specifically with ejaculation (see the section An Ejaculation-Related Circuit later in the chapter). Co-localization of Fos-IR with either glutamate or glutamic acid decarboxylase (GAD),

the enzyme that produces GABA, revealed that half of the activated cells in the lateral MePD were GABAergic, while a quarter were glutamatergic.[380] Finally, Fos-IR in the Me, but not in the MPOA, was positively correlated with the duration of the postejaculatory interval, suggesting that this area may contribute to postejaculatory quiescence.[11]

As determined by co-localization of Fos with steroid receptors, many hormone-sensitive neurons in the Me are activated during mating. In male rats and hamsters, AR-containing neurons in posterior Me express Fos after mating. Furthermore, neurons that co-localize Fos-IR and AR-IR are interconnected, as determined in tract tracing studies (see the section An Ejaculation-Related Circuit later in the chapter). On the other hand, castration does not appear to reduce Fos in the Me and other central chemosensory targets of male rats[381] or hamsters.[336] Likewise, the pattern of Fos expression in Me and MPOA was similar in wild-type mice and in those lacking the aromatase gene.[382] Therefore, gonadal steroids can have a long-term permissive action on mating behavior circuitry, but are not required acutely for Fos activation.

Effects of Hormone Implants

As noted above, the Me is sensitive to gonadal steroid hormones. ARs and ERs are concentrated in the posterior subdivision of the Me (reviewed in Ref. 3). This subnucleus and the encapsulated area of the bed nucleus of the stria terminalis (BST) are twice as large in males as in females, and contain more substance P, CCK, and vasopressin.[383] Likewise, the posteromedial cortical amygdaloid nucleus is also larger in males.[384] The MePD also shows a different pattern of synaptic contacts, compared with females (reviewed in Ref. 3). Castration in male rats[104] caused the MePD to shrink; however, androgen treatment of adult female rats increased the MePD to male-typical size, by enlarging soma size. Therefore, hormone treatment in adulthood can completely reverse a sexual dimorphism in a brain area important for sexual behavior.

Hormone implants in the Me can facilitate the maintenance or restoration of male sexual behavior. Bilateral T implants into the Me of castrated male rats delayed the loss of both copulation and NCE.[385] Similarly, either T[386] or E2[387] implants into the Me restored copulatory behavior in castrated male hamsters. Likewise, E2 stimulated sexual activity in male rats,[388] including those treated systemically with an aromatase blocker.[389] However, DHT implants were ineffective in facilitating sexual behavior, suggesting that stimulation of ARs in the Me is not sufficient for behavioral restoration in male hamsters.[386] On the other hand, DHT implants into the Me were sufficient for behavioral activation in castrated rats receiving subthreshold systemic injections of E2.[390] Implants of the AR antagonist hydroxyflutamide into the Me of T-replaced castrated male rats partially inhibited copulation,[391] but the E2 antagonist tamoxifen likewise failed to block mating in castrated male hamsters with systemic T replacement.[392] Therefore, stimulation of ARs in the Me contributes to male copulatory behavior in

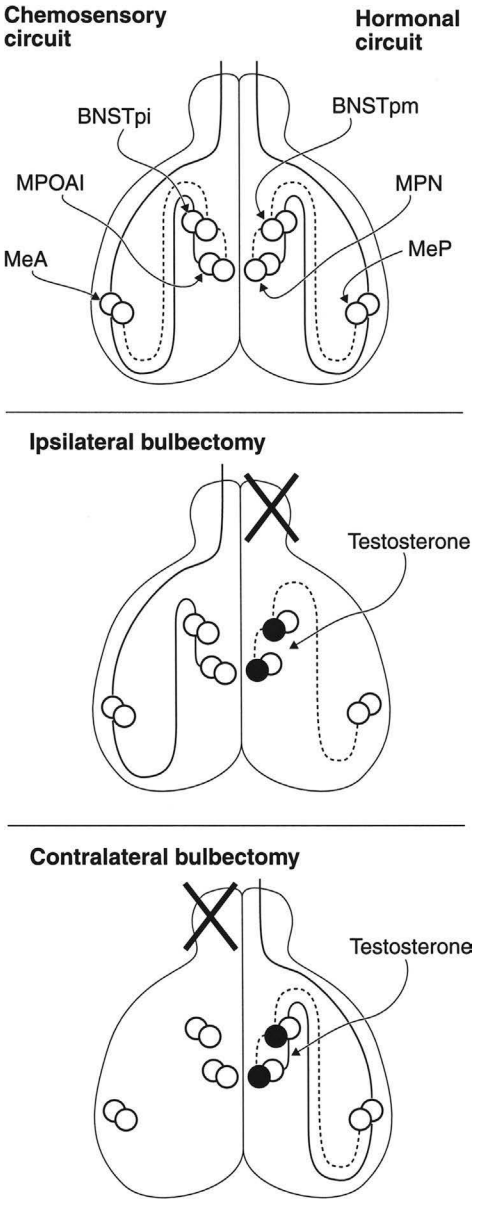

FIGURE 49.12 Schematic diagram of mating behavior circuitry through the nucleus of the medial amygdala (Me), bed nucleus of the stria terminalis (BNST), and medial preoptic area (MPOA) in the male Syrian hamster brain, illustrating the separate pathways for receipt of chemosensory and hormonal cues. Top, Normal male. Elements of the chemosensory circuit (left) include the anterior subdivision of the Me (MeA), posterointermediate subdivision of BNST (BNSTpi), and the lateral subdivision of MPOA (MPOAl). The hormonal circuit (right) consists of the posterior subdivision of the Me (MeP), the posteromedial subdivision of BNST (BNSTpm), and the medial preoptic nucleus (MPN). Middle, Testosterone stimulation of BNSTpm and MPN (solid circles) combined with removal of the ipsilateral olfactory bulb prevents communication between the chemosensory and hormonal circuits. Bottom, Removal of the contralateral olfactory bulb permits chemosensory and hormonal integration. *Source: Figure is reprinted from Ref. 393, with permission.*

both rats and hamsters. However, androgens in Me are not necessary, and may not be sufficient for copulation in male rats or hamsters.

Because the Me receives chemosensory and hormonal stimuli, both of which are essential for mating in male hamsters, the interactions between hormonal and chemosensory stimulation were probed. Olfactory bulbectomy, either ipsilateral or contralateral to a T implant into the Me, rendered the implant ineffective (see Figure 49.12).[387] Similar implants into the MPOA were less susceptible to the loss of olfactory input.[393] Cottingham and Pfaff[394] suggested that steroid-responsive networks should exhibit redundancy, amplification, stability, and selective filtering. Because both the Me and the MPOA transduce steroid cues and receive chemosensory input, Coolen and Wood[395] tested whether steroid implants into these two areas exhibit steroid amplification. Although there is some redundancy, in that steroid implants into either area were sufficient to activate copulation, there appears to be no amplification, in that males with dual implants into the Me and MPOA showed no greater copulatory ability than did males with single implants.

Microinjections of Drugs

Microinjection of angiotensin II into the Me impaired sexual behavior in male rats.[396] This effect was prevented by antagonists to both angiotensin I and II receptors. Therefore, both angiotensin I and II receptors may contribute to angiotensin II's inhibitory effects on copulation. Angiotensin II[397] and its AT1 receptor[398,399] are found in the amygdala of male rats.

Amygdaloid Efferents

Efferents from the corticomedial amygdala travel via the stria terminalis and ventral amygdalofugal pathway to innervate the BST, MPOA, VMH, and other midline hypothalamic nuclei.[400] In male hamsters, cutting the stria terminalis increased mount and ejaculation latencies and interintromission interval.[401] Knife cuts of the ventral pathway produced a less severe loss of mating; however, combined knife cuts of both the strial and ventral projections of the corticomedial amygdala eliminated copulation.[401] Similar pathways may contribute to male sexual behavior in rats (reviewed in Ref. 3).

Through the stria terminalis and ventral pathway, the anterior and posterior subdivisions of the Me maintain distinct, yet parallel connections with subnuclei of the BST. While the posterior subnucleus of Me projects to the posteromedial BST, the anterior Me sends projections preferentially to the intermediate subdivisions of posterior BST.[402] These connections are part of the extended amygdala, a ring of interconnected nuclei linking the central Me with the BST.[403]

Bed Nucleus of the Stria Terminalis

Like the amygdala, the bed nucleus of the stria terminalis (BST) is a heterogeneous brain region with functionally and anatomically distinct subnuclei (reviewed in Ref. 404). The posteromedial regions of the BST are particularly relevant to male mating behavior.[405] As part of the extended amygdala, the Me and BST share overlapping functions and connections; in particular, the posteromedial BST and posterior Me each have abundant steroid hormone receptors (reviewed in Ref. 3). Anatomically, the BST is intermediate between the Me and MPOA; however, chemosensory cues transmitted through Me can also bypass the BST to reach the MPOA directly.

Effects of Lesions

Lesions of the BST did not block mating in male rats or hamsters; however, they reduced NCE, increased the number of intromissions preceding ejaculation, and slowed copulation in rats (reviewed in Ref. 3). These lesions did not affect intromission ratio in rats, suggesting that *in-copula* erections were not affected. These results are in contrast to the effects of lesions of the MPOA, which had little effect on NCE, but dramatically impaired copulation.[406] In male hamsters, large lesions of BST did prevent copulation, but smaller lesions of the posteromedial BST reduced anogenital investigation without preventing ejaculation.[407]

In male gerbils, bilateral lesions of either the caudal medial BST or the sexually dimorphic area (SDA) of the preoptic area severely impaired copulation.[408] Furthermore, unilateral lesions of the caudal medial BST and the contralateral SDA also decreased copulation; ipsilateral lesions had much less effect. Therefore, the caudal medial BST appears to perform an important integrative function. It is not clear whether the different effects of BST lesions in rats, hamsters, and gerbils reflect species differences or differences in experimental procedures.

Activation of c-fos *or Other Immediate–Early Genes*

Exposure to a sexually relevant olfactory stimulus, NCE, or mating increased Fos-IR in the BST of male rats[340] and hamsters.[333] In common with effects in the Me, exposure to estrous odors, but not sexually conditioned almond odor, increased activity in the BST of male rats.[354]

The pattern of mating-induced Fos expression in BST is similar to that for Me, with increases in BST, especially in the posterior and medial subdivisions, in rats, hamsters, and gerbils (reviewed in Ref. 3). By contrast, mating reduced Fos in the BST of male macaques.[409] In rodents, exposure to chemosensory cues from estrous females or their bedding also stimulated Fos, although mating

to ejaculation induced further increases (reviewed in Ref. 3). In contrast, there was no increase in Fos-IR in the BST of male ferrets exposed to estrous female bedding.[344] In hamsters, Fos induction in posteroventral BST and MPOA appears to be selective for mating and sexual stimuli, because aggression failed to induce Fos in these regions.[370]

Hormonal Effects

Posteromedial BST has abundant steroid receptors that extend ventromedially from the stria terminalis to the medial preoptic nucleus.[410–412] Castration decreased immunoreactivity for CCK in the encapsulated portion of BST.[413] T treatment restored CCK to normal levels. Similar effects were observed in sexually dimorphic areas of the Me and medial preoptic nucleus.

Central Tegmental Field/Subparafascicular Nucleus of the Thalamus

The midbrain tegmentum is reciprocally connected to the MPOA, Me, and anterior hypothalamus (reviewed in Ref. 3). Subregions of the midbrain tegmentum have been referred to as the central tegmental field (CTF) or the dorsolateral tegmentum (DLT). This region lies immediately dorsal to the lateral half of the substantia nigra and includes the subparafascicular nucleus, part of the caudal zona incerta, the peripeduncular nucleus, the mesencephalic reticular nucleus, and the anterior pretectal nucleus (see Figure 49.13). In particular, the medial parvocellular portion of the subparafascicular nucleus (SPFp) relays somatosensory cues from the penis and perineum to the MPOA and Me. The SPFp is part of an ejaculation circuit in rats, hamsters, and gerbils (see the section Circuitry and Anatomical Interconnections later in the chapter).

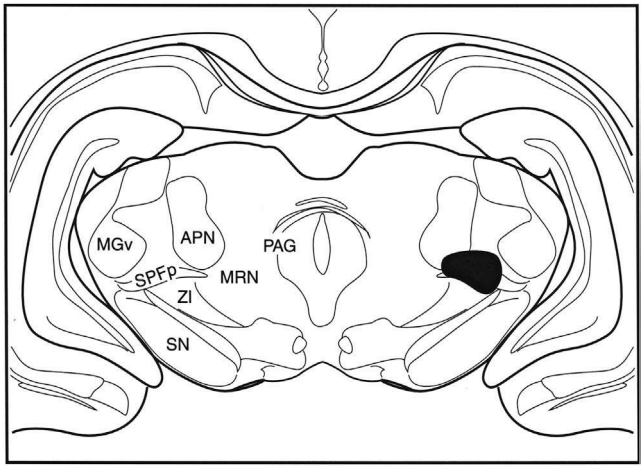

FIGURE 49.13 Site of fluorogold (FG) injection into the lateral central tegmental field (CTF); black area on right side of section. The site of injection was defined as the area of brilliant FG surrounding the region of necrosis induced by the injection. APN, anterior pretectal nucleus; MGv, venral medial geniculate nucleus of the thalamus; MRN, median raphe nucleus; PAG, periaqueductal gray; SN, substantia nigra; SPFp, parvocellular portion of the subparafascicular nucleus; ZI, zona incerta. *Source: Figure is redrawn from Ref. 414, with permission.*

Effects of Lesions

Bilateral lesions of the CTF virtually eliminated mating behavior of male rats, as well as sniffing, pursuit, self-grooming, and genital exploration of the female.[415] Similarly, bilateral lesions of either the MPOA or the CTF, or unilateral lesions of the MPOA and contralateral CTF, inhibited both copulation and the preference for an estrous female,[416] suggesting that connections between these two areas are essential for copulation.[415]

Subsequent lesion studies emphasized the importance of the CTF in general, and the SPFp in particular, in transmitting ascending genitosensory information to the MPOA. Combined ipsilateral excitotoxic lesions of the CTF and Me in male rats abolished copulation-induced Fos-expression in the MPOA.[337] Unilateral lesions of either the CTF or Me alone did not affect Fos in the MPOA. Likewise, unilateral MPOA lesions did not affect Fos in the CTF or Me. In gerbils, bilateral lesions of the SPFp did not affect copulation, suggesting that ejaculation-induced Fos expression represents a response to ejaculation-related stimuli, rather than participation in the control of ejaculation.[358] However, tract tracing studies with male rats show that the SPFp receives projections from lumbar spinothalamic neurons,[417] which are essential for sexual behavior[43] and express ejaculation-induced Fos.[418]

Expression of c-fos *or Other Immediate–Early Genes*

Ejaculation selectively increased Fos in the SPFp of rats, gerbils, hamsters, and musk shrews (reviewed in Ref. 3). Fos was not induced by chemosensory investigation, mounts, or intromissions. In human male volunteers, ejaculation stimulated cerebral blood flow in SPFp as measured by positron emission tomography.[419] Likewise, electrical stimulation of CTF accelerated mating in male rats.[420]

To test whether SPFp Fos expression was selective for ejaculation, Coolen et al.[421] administered the 5-HT_{1A} agonist, 8-OH-DPAT, to male rats to decrease the number of intromissions preceding ejaculation. Indeed, some animals ejaculated on their first intromission. Males that ejaculated after few or no mounts or intromissions had almost as many Fos-IR neurons in the SPFp as did those that ejaculated following the normal numbers of mounts and intromissions. Furthermore, male and female rats have very similar patterns of Fos-IR following ejaculation, again suggesting the importance of ejaculation-related stimuli rather than motor control.[365]

Presence of Steroid Receptors

SPFp neurons have ARs,[422] and many androgen-responsive neurons in SPFp that project to MPOA express Fos following ejaculation.[373]

Connections

The SPFp consists of medial and lateral subdivisions. Neurons of the lateral subdivision express calcitonin gene-related peptide and transmit visual and auditory stimuli for conditioned fear.[423] The medial portion of the SPFp contains a high concentration of galanin-IR fibers.[365] Mating induces Fos in the medial subdivision, and Fos-positive nuclei are surrounded by galanin-IR fibers. Medial SPFp receives projections from neurons in laminae 7 and 10 of the lumbosacral spinal cord and has bidirectional connections with forebrain nuclei controlling sexual behavior.[417] Many of these connections also contain AR and mating-induced Fos.[373] Thus, galanin may be used to relay ejaculation-related somatosensory information through SPFp.

CENTRAL INTEGRATIVE AREAS

Medial Preoptic Area

The medial preoptic area (MPOA) is a critical integrative site for male sexual behavior in all vertebrate species that have been tested. This is remarkable considering the range of sensory stimuli that elicit mating and the diversity of motor patterns that express copulation. Such behavioral flexibility is possible because the MPOA receives indirect input from every sensory modality and sends reciprocal connections back to those sources.[424] These reciprocal connections allow the MPOA to modify the processing of sensory input. Furthermore, the MPOA and its principal afferents contain steroid receptors, which provide the means to bias sensory input to favor sexually relevant stimuli. Efferent connections from the MPOA are critical for the initiation and patterning of copulation. They project to hypothalamic, midbrain, and brainstem nuclei that regulate autonomic or somatomotor patterns and motivational states (reviewed in Ref. 425).

Like other elements of the circuitry for male sexual behavior (e.g., Me and BST), the MPOA is a heterogeneous region comprising separate subnuclei, each with a distinct profile of functions, connections,[424] and neurotransmitter content.[424] The MPOA includes a medial periventricular zone chiefly concerned with neuroendocrine regulation, and a medial zone that functions in male sexual behavior and maternal behavior. Subnuclei within the MPOA are heavily interconnected. In particular, there is potential for reciprocal interaction of sexual behavior and neuroendocrine function via connections between nuclei of the medial and periventricular zones. Within the medial zone of the MPOA, the medial preoptic nucleus (MPN) and posterodorsal preoptic nucleus (PdPN) are particularly important for sexual behavior. Using a variety of experimental approaches, it appears that the MPOA is essential for copulation, and contributes to sexual motivation as well.

Effects of Lesions

Lesions of the MPOA severely impair copulation in male rats, hamsters, mice, guinea pigs, gerbils, dogs, cats, ferrets, goats, marmosets, rhesus monkeys, birds, lizards, snakes, and fish (reviewed in Ref. 3). Large lesions of the MPOA, extending into the rostral anterior hypothalamus, eliminated copulation in sexually experienced male rats; indeed, none of the lesioned males mounted a receptive female rat in periodic tests up to 3[426] or even 8 months[427] after surgery. It is important to note that similar results were obtained using either electrolytic lesions or axon-sparing excitotoxic lesions. Inactivation of the MPOA by lidocaine also impairs motivation, as well as copulation.[428] Therefore, neurons within the MPOA play a critical role in both sexual motivation and the activation of copulation.[429]

A number of attempts have been made to reactivate copulation in male rats that have MPOA lesions. In the early studies of Heimer and Larsson,[426] stimulation of lesioned males by handling or exposure to different females failed to elicit copulation. Chronic T administration was similarly ineffective, an indication that the effects of the MPOA lesions were not an indirect result of diminished gonadal output. Even more extreme forms of excitation, such as repeated flank shock, could not compensate for MPOA damage.[430] However, systemic injection of lisuride, a nonspecific monoamine receptor agonist, transiently activated copulation in 100% of male rats with MPOA lesions, with 50% copulating to ejaculation.[429] When these animals were subsequently treated with saline, they once again failed to copulate. Under similar conditions, administration of GnRH[267] or naloxone[429] was ineffective.

Smaller lesions of the MPOA typically produced less severe deficits. In marmosets, lesions confined to the MPOA decreased copulation, while lesions at the junction of the MPOA and anterior hypothalamus eliminated intromission and ejaculation.[431] Similarly, in rats, lesions placed in rostral MPOA produced less severe copulatory deficits, with many of the males copulating to ejaculation; males that were still able to copulate after small preoptic lesions had prolonged intromission and ejaculation latencies, were inconsistent from test to test, and in some cases recovered normal copulatory ability over time (reviewed in Ref. 3). Small lesions targeting the posterodorsal preoptic nucleus, which is selectively activated by ejaculation in male gerbils,[374] decreased mounting and delayed ejaculation, suggesting that this

area normally facilitates copulation.[358] By contrast, damage to caudal MPOA, which included the rostral anterior hypothalamus, was associated with a severe copulatory deficit.[432] In male hamsters, lesions of caudal MPOA centered in the magnocellular region of the medial preoptic nucleus eliminated copulation.[407]

In rats and gerbils, the MPOA has sexually dimorphic subregions that are attractive candidates to regulate male sexual behavior. In gerbils, the sexually dimorphic area consists of three interconnected subnuclei that are part of a critical circuit for the control of male sexual behavior (reviewed in Ref. 433). Unilateral excitotoxic lesions of the medial SDA, paired with similar lesions of the contralateral lateral SDA, were as effective as bilateral lesions of each.[434] Conversely, Arendash and Gorski[435] found that lesions of the sexually dimorphic nucleus of MPOA (SDN-POA) in the male rat had no effect on copulation in experienced rats, and produced only a transient mating delay in sexually naive males.[436] However, lesions dorsal to SDN-POA reduced the percentage of male rats that ejaculated.[435] Also, perinatal administration of an aromatase inhibitor decreased the size of the SDN-POA and inhibited copulation in adulthood, and there was a significant correlation between the size of the SDN-POA and males' preference for a female partner rather than a male.[437]

It was surprising that MPOA lesions in juvenile male rats reared in isosexual or heterosexual groups produced only minimal copulatory deficits when these animals were tested as adults.[438,439] The only discernable effects were that lesioned males had fewer ejaculations before sexual exhaustion and showed a more rapid decline in copulation following castration.[438] In contrast, males lesioned as juveniles and reared in social isolation did not copulate as adults.[439] However, similar detrimental effects of social isolation in male rats without brain damage have also been reported (e.g., see Ref. 440). Furthermore, copulation was observed in male rats that received lesions as juveniles and were socially isolated, but were handled daily until adulthood,[441] suggesting that interactions with conspecifics are not critical for the development of copulatory behavior in these animals. Another study in rats reported incomplete behavioral recovery following MPOA lesions in juvenile male rats.[441] However, the MPOA lesions in these studies were larger and more caudal, and would be expected to produce the most severe deficits in adult male rats. Thus far, juvenile plasticity in copulatory behavior after MPOA lesion seems to be specific to rats, as MPOA lesions in juvenile dogs[442] and cats[443] eliminated copulation, despite extensive social experience.

It is significant that large MPOA lesions eliminate not only ejaculation, but also the initiation of copulation. This has been interpreted as a reduction in sexual arousal or motivation (e.g., see Refs 416,427,444). However, male rats with MPOA lesions pursued estrous female rats and investigated their anogenital region, climbed over them, and occasionally clasped and mounted them without thrusting.[426,430,445] A similar pattern of behavior has been described for male cats and dogs with MPOA lesions. Indeed, male dogs with MPOA lesions mounted and sometimes showed shallow thrusting, but did not copulate effectively.[446,447] Although monkeys or rats with preoptic area lesions rarely showed female-directed sexual contact, they would press a bar for access to a receptive female or for a secondary reinforcer that had previously been associated with a receptive female (reviewed in Ref. 353). Furthermore, the frequency of masturbation by the monkeys was unaffected by MPOA lesion; although they were clearly sexually aroused, they did not appear to recognize or respond to the female as a sexual partner. Similarly, MPOA lesions that dramatically impaired copulation had little or no effect on NCE.[406] Based on this apparent dissociation between sexual motivation and copulatory performance, Everitt[353] suggested that the MPOA controls only copulatory performance and does not influence sexual motivation.

However, lesions of the MPOA have diminished sexual motivation in other contexts, including the preference for a female partner in rats and ferrets, pursuit of a female by male rats, or precopulatory behavior (anticipatory erections, tongue flicking, and anogenital investigations) in marmosets (reviewed in Ref. 3). Therefore, while sexual motivation is not eliminated by MPOA lesions, it is clearly diminished. The evolutionary conservation of MPOA influence on both sexual motivation and performance is apparent in studies of Japanese quail and starlings. In quail, small lesions of subregions of the MPOA differentially impaired copulatory performance and sexual motivation, measured as time spent in front of a window through which the male could view a female.[448] In starlings, MPOA lesions selectively reduced singing and the gathering of green nesting materials.[226]

The specificity of effects of MPOA lesions is highlighted by contrast with the effects of lesions in the lateral preoptic area (LPOA). Like humans, rats have erections during episodes of paradoxical sleep.[449] Schmidt et al.[450] showed that rats with MPOA lesions experience no disruption of sleep or of erections during sleep. In contrast, LPOA lesions dramatically reduced the occurrence of erection during paradoxical sleep, whereas erections during the waking state were unchanged. The reduction in erections was not attributable to impaired paradoxical sleep, although males with LPOA lesions had extended insomnia and less slow-wave sleep. Although most of the affected males also had some damage to the ventral BST, case analysis suggested that it was LPOA damage that contributed most to the observed effects. This study also exemplifies the context-specificity of the regulation of erection.

Effects of Electrical or Chemical Stimulation

As expected from the inhibitory effects of lesions, stimulation of the MPOA has resulted in facilitation of copulation in male rats, guinea pigs, and opossums (reviewed in Ref. 3). The typical effects included decreases in mounts and intromissions preceding ejaculation, ejaculation latency, and postejaculatory intervals. Lesions of the medial forebrain bundle, which carries the major efferents from the MPOA, diminished the effects of stimulation in opossums.[451] Indeed, stimulation of the hypothalamic area that encompasses the medial forebrain bundle produced facilitation of the same measures as MPOA stimulation (reviewed in Ref. 3).

Electrical stimulation of the MPOA failed to restore copulation in male rats 24 h after they had copulated to sexual satiety, although it facilitated several measures in nonsatiated males.[452] Furthermore, combining the electrical stimulation with subthreshold doses of the DA agonist apomorphine or the α_2-adrenoceptor antagonist yohimbine was also ineffective in satiated males. Higher doses of both drugs had previously restored copulation in satiated animals.[138] The authors suggested that different mechanisms may underlie sexual satiety and the postejaculatory interval, and that stimulation of an excitatory site is not sufficient to reverse sexual satiety.

Repeated electrical stimulation (four per day, separated by 2 h) of the MPOA of previously noncopulating rats led to "kindling" (increased after-discharges, leading eventually to a seizure) and the subsequent ability of most males to copulate on tests without stimulation.[453] Males that initially copulated without stimulation were not further facilitated. Therefore, kindling in the MPOA decreased the threshold for initiating copulation but did not affect normal copulatory performance. Kindling-like stimulation that did not result in seizure activity resulted in permanent facilitation of copulation in previously noncopulating rats, even after 8 months.[454] Similarly, MPOA kindling in T-treated female rats induced male-like copulatory behavior.[455]

Electrical stimulation of the MPOA or medial forebrain bundle has also elicited ex copula erections and/or seminal emissions in monkeys and rats; however, other researchers, using different stimulation parameters, reported erections only following the end of stimulation trains (reviewed in Ref. 3). Either electrical or glutamatergic stimulation of the MPOA elicited increases in intracavernous pressure in anesthetized rats[456,457] and also elicited the UG reflex, even without genital stimulation.[458] The effects of MPOA stimulation were enhanced by intrathecal application of an NO donor, a cGMP analog, and sildenafil, and were decreased by the NOS inhibitor L-NAME.[457] MPOA neurons do not project directly to the lower spinal cord, where genital reflexes are controlled; therefore, MPOA efferents must activate downstream sites that in turn regulate erection and seminal emission. Furthermore, the MPOA appears not to be necessary for penile erection or seminal emission, since MPOA lesions have had little effect on NCE,[406] touch-based erections,[459] or spontaneous seminal emission.[460]

Effects of Direct Applications of Steroids or Steroid Antagonists

In view of the substantial numbers of steroid receptors in the MPOA, it is not surprising that intracerebral application of T facilitates mating in castrated males. Fisher[461] first tested brain localization of T effects on copulation, using a water-soluble derivative of T. Although detailed methods and data are not available, Fisher indicated that the preoptic area was the most effective site for the activation of copulation in castrated male rats. A more detailed study, using crystalline T implants, reported that 100% of the males receiving MPOA implants copulated to ejaculation.[462] Cholesterol control implants were ineffective, and T implants in hypothalamic, hippocampal, and "other" sites yielded 0–81% of animals copulating to ejaculation. The effectiveness of T implants in the MPOA has been confirmed in studies of castrated male rats, ferrets, birds, and anole lizards (reviewed in Ref. 3). Therefore, androgenic mechanisms in the MPOA that stimulate copulation are highly conserved in evolution.

Nonetheless, stimulation of sexual activity in castrated males by intracerebral steroid implants is variable. For instance, in one study, although 100% of the males implanted with T in the MPOA copulated to ejaculation[462] these males ejaculated in only 57% of the tests. Even among males that copulated to ejaculation, mating behavior was not always normal (e.g., see Ref. 463). Similar observations have been reported for male hamsters and mice (reviewed in Ref. 3). Intracerebral T implants in mice increased ultrasonic vocalizations, preference for a female, and urine marking in response to female urine; however, little copulation was observed.[464] Most likely, this is due to selective stimulation of only a limited portion of the male mating behavior circuitry, and the absence of systemic androgens. In this regard, combined implants of T in the MPOA and Me did not further stimulate sexual activity above that induced by single MPOA implants in male mice[465] or hamsters.[395]

Estradiol in the MPOA stimulated sexual activity in castrated male rats[466] and hamsters.[467] Some investigators have not observed ejaculation with E2 implants.[468] However, when Christensen and Clemens[469] compared the efficacy of T or E2 in the MPOA to activate copulation, only 30% of T-implanted males copulated to ejaculation, whereas 70% of males implanted with E2 ejaculated. In a subsequent study, infusion of T or E2 into the rostral anterior hypothalamus stimulated mounting.[466] However, concurrent treatment with ATD (an aromatase

inhibitor) blocked the increase in mounting induced by T, without affecting E2-stimulated mounts. In complementary studies, the nonsteroidal aromatase inhibitor fadrozole impaired sexual behavior when delivered into the MPOA[470] or the lateral ventricle.[99] Moreover, Clancy et al.[471] implanted E2 into the MPOA of gonadally intact males treated systemically with an aromatase inhibitor. Thus, E2 was available only to the MPOA, although androgens were available throughout the body. Estradiol-treated animals regained about half the numbers of mounts, intromissions, and ejaculations that they had exhibited before surgery, while copulatory behavior of controls remained low. Therefore, E2 in the MPOA combined with systemic androgen is sufficient to maintain at least some copulatory and ejaculatory behavior. Collectively, these results are consistent with the idea that E2 is able to stimulate copulation in males, and that the effects of T require aromatization to E2 within cells of the central nervous system.

The aromatization of T to E2 in the MPOA may also be important for sexual preference in rams. Rams that prefer to mate with other rams have reduced capacity to aromatize T to E2 in the MPOA and anterior hypothalamus, in addition to lower circulating levels of T and E2.[472] High sexually performing rams also had more ERs than did low sexually performing rams.[473]

There have been relatively few experiments testing implants of DHT in the MPOA to activate male sexual behavior. DHT implants in the MPOA and anterior hypothalamus induced copulation in only 33–35% of castrates, a percentage not significantly different from cholesterol-treated controls.[463,468] For the DHT-treated males that copulated to ejaculation, intromission latency and the number of mounts to intromission were significantly elevated compared with precastration levels, although other measures of copulation were normal.[463] Implantation of DHT into the anterior hypothalamus of male hamsters had no detectable effect on copulation.[467] Baum et al.[390] were somewhat more successful in stimulating copulation with DHT implants in the MPOA/anterior hypothalamus of male rats. In this study, castrated males ejaculated on about 35% of the tests prior to implantation, whereas the percentage increased to about 70% after the males received intracranial DHT. Likewise, implants of the anti-androgen flutamide into the MPOA blocked sexual behavior in castrated rats treated systemically with T.[474] Male guinea pigs have been shown to be particularly responsive to intrapreoptic area implants of DHT, with full copulatory behavior restored in virtually all males tested.[475]

Another approach to study the effects of intracranial steroid administration on copulation has been to combine a brain implant with systemic hormone treatment subthreshold for the elicitation of behavior. In one study, MPOA implants of DHT combined with subthreshold systemic DHT treatment in castrated rats promoted ejaculation in 40% of males.[476] When Davis and Barfield[463] gave brain implants of E2 to castrated male rats treated systemically with DHT, 60% copulated to ejaculation. Baum et al.[390] performed the complementary experiment by implanting DHT into the brain of male rats receiving systemic E2. DHT implants in the MPOA/AH were ineffective, but DHT did stimulate mating when implanted in the lateral septum or Me.

Yet another approach is to block either steroid receptors or enzymes that produce E2 or DHT locally. Fadrozole, a nonsteroidal aromatase inhibitor, delivered into either the MPOA[470] or the lateral ventricle,[99] impaired copulation in male rats. In contrast, an implant of E2 in the MPOA partially reversed the impairment by systemic administration of fadrozole.[471] These studies support the importance of E2 in the MPOA for male sexual behavior. However, MPOA androgen also contributes to sexual behavior. Administration of the antiandrogen flutamide into the anterior MPOA impaired copulation, but not sexual motivation (partner preference), whereas flutamide in the posterior MPOA had the reverse effects: impairment of motivation, but not copulatory performance.[477]

Effects of Direct Application of Drugs Affecting Specific Transmitters

The MPOA plays a central role in coordinating many facets of sexual behavior, so it is not surprising that drugs administered into the MPOA have affected numerous behavioral measures. Most effects of locally administered drugs resemble those of the same drug injected systemically. However, there are a few site-specific differences.

DOPAMINE

MPOA DA facilitates copulation, genital reflexes, and sexual motivation. Dopaminergic neurons of the periventricular system lie along the third ventricle and send widely branching axons laterally into the MPOA and anterior hypothalamus (reviewed in Ref. 477a). The classic D_1/D_2 agonist apomorphine, microinjected into the MPOA, facilitated copulation in intact male rats[478] and in long-term castrates,[143] and also increased the numbers of touch-based erections and seminal emissions.[479] Similarly, reverse-dialysis of the DA reuptake inhibitor bupropion into the MPOA increased levels of extracellular DA and increased both touch-based and NCE.[480] Conversely, the D_1/D_2 antagonist *cis*-flupenthixol in the MPOA impaired copulation and genital reflexes, and decreased the number of choices of a female in an X-maze, without affecting running speed or the number of no-choice trials (reviewed in Ref. 3). The decrease in sexual motivation (choice of the female) was relatively modest, probably because the drug was injected

only unilaterally. However, there was spatial specificity with both apomorphine's and *cis*-flupenthixol's effects; microinjections anterior, dorsal, or lateral to the MPOA were ineffective in all experiments. There was also behavioral specificity, in that neither apomorphine nor *cis*-flupenthixol affected eating, drinking, locomotion, or rearing in the home cage, although flupenthixol slightly increased time spent inactive.[481] MPOA microinjections of a different DA antagonist, haloperidol, also resulted in increased intromission latency and decreased ejaculations in a bilevel apparatus, although motoric slowing may have contributed to these deficits.[482] In a more recent study, microinjection of apomorphine into the MPOA of males whose copulatory ability had been severely compromised by large excitotoxic lesions of the amygdala, fully restored their copulatory ability.[355] Trends toward apomorphine-induced increases in intromissions and ejaculations in sham animals were not statistically significant. These data suggest that a major consequence of amygdala lesions is impaired DA activity in the MPOA.

Studies found that 6-OHDA neurotoxic lesions of DA neurons in the MPOA impaired copulation only within the first 24 h after the lesion[483] or if a subthreshold dose of a DA synthesis inhibitor was administered.[484] There was only a 23% depletion of DA, a likely result of the sparse population of DA transporters in the MPOA, which are necessary to transport the toxin into the axon terminals. Thus, increased DA synthesis in the remaining neurons occurred during the first 24 h after the lesion and was able to restore copulation to normal. This effect was specific for DA, since the 6-OHDA was co-administered with desipramine, an NE transporter inhibitor; and NE levels were not affected.

Stimulation of D_1 receptors has consistently facilitated copulation and touch-based erections, whereas D_2 agonists have produced biphasic effects. A low dose of the D_2/D_3 agonist quinelorane, microinjected into the MPOA, decreased the latency to the first genital reflex, without affecting the numbers of erections, anteroflexions, or seminal emissions.[485] Thus, small increases in dopaminergic stimulation may disinhibit reflexes via D_2-like receptors. However, a large dose of quinelorane, or of the D_1 antagonist SCH-23390 decreased erections but increased seminal emissions. Thus, intense stimulation of D_2-like receptors or antagonism of D_1-like receptors appear to facilitate sympathetically controlled seminal emission while inhibiting parasympathetically controlled erections. Conversely, a D_1 full agonist, tetrahydrothienopyridine (THP) increased touch-based erections but inhibited seminal emission.[486] The D_1 partial agonist SKF-38393 was ineffective (unpublished observations). Experiments using low and high doses of apomorphine, together with D_1 and D_2 antagonists, also support the opposing effects of D_1 and D_2 receptors on autonomic function. The low dose of apomorphine (1 μg) increased touch-based erections, but not seminal emissions; this effect was blocked by both D_1 (SCH-23390) and D_2 (raclopride) antagonists, suggesting that both disinhibition by D_2 receptors and facilitation by D_1 receptors contributed to apomorphine's increase.[487] Unexpectedly, a high dose (10 μg) of apomorphine failed to increase erections significantly; however, co-administration of the high dose with a D_2 antagonist unmasked the facilitative effects apparently mediated by D_1 receptors. The high dose of apomorphine, administered alone, did increase seminal emissions, an effect that was blocked by a high dose of the D_2 antagonist, but not by the D_1 antagonist. According to a more recent study, it is the D_3 receptor, a member of the D_2 family, that promotes ejaculation. A D_3 agonist (7-OH-DPAT) microinjected into the MPOA of anesthetized rats elicited rhythmic contractions of the bulbospongiosis muscle, seminal vesicle pressure increases, and/or semen expulsion; this response was mostly abolished by simultaneous MPOA microinjection of a D_3 antagonist (nafadotride).[488]

The association of D_1 receptor activation in the MPOA with erections, and of intense D_2/D_3 stimulation with ejaculation, was supported in copulation tests. The D_1 agonist THP, microinjected into the MPOA, increased copulatory efficiency, resulting in more ejaculations per 30-min test.[486] Conversely, the D_1 antagonist SCH-23390 increased intromission latency but decreased the threshold for ejaculation.[489] It also decreased the choice of the female in an X-maze, without impairing motor activity.[490] A high dose of the D_2/D_3 agonist quinelorane also increased intromission latency and decreased ejaculatory threshold.[490] However, it did not decrease the percentage of X-maze trials on which the male chose the female, but rather increased the latency to reach the female's goal box.[490] This delay was not the result of slowed motor function, but of apparent reluctance to cross the line in front of the female's compartment, beyond which the male would be placed into that compartment. In summary, D_1 and D_2 receptors in the MPOA appear to exert reciprocal effects on copulation and genital reflexes. Activation of D_1 receptors facilitates parasympathetically mediated erections and copulatory performance, whereas activation of D_2 receptors promotes sympathetically mediated ejaculation and the postejaculatory state.

Activation of D_1 receptors also facilitates the experience-induced enhancement of mating in male rats[491] (Figure 49.14). Microinjection of a D_1 antagonist (SCH-23390) into the MPOA before each of seven noncopulatory exposures to an estrous female over the male's cage dose-dependently blocked the facilitative effects of such exposures on a subsequent drug-free mating test, compared to effects of vehicle injections. In addition, there was greater phosphorylation of DARPP-32 (dopamine- and cAMP-regulated phosphoprotein), a downstream marker of D_1 activation, in males that had both chronic

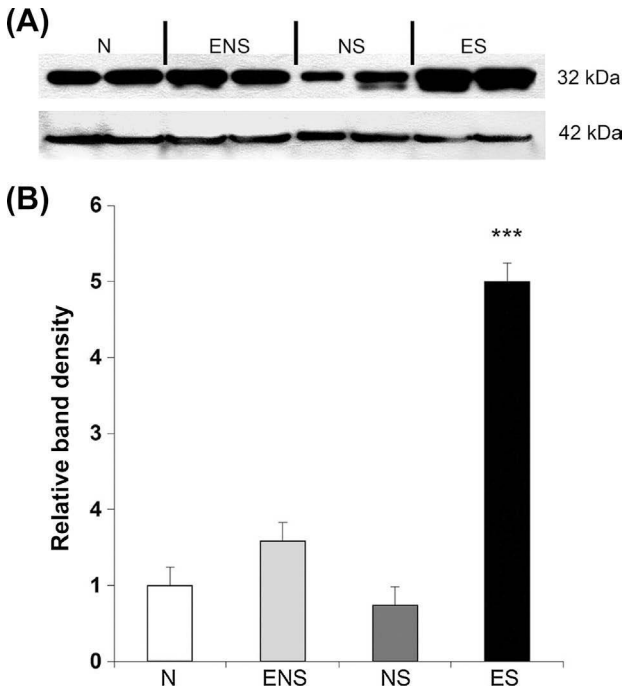

FIGURE 49.14 Protein concentration for phosphorylated dopamine- and cAMP-regulated phosphoprotein (pThr34-DARPP-32) in the medial preoptic area (MPOA) of male rats. (A) Western immunoblot of samples collected from the MPOA of male rats that were either sexually naive and did (NS) or did not (N) mate on the day of euthanasia and sexually experienced rats that did (ES) or did not (ENS) mate on day of euthanasia. The immunoblots depict bands for pThr34-DARPP-32 at 32 kDa and bands for beta-actin at 42 kDa. (B) Graph summarizing relative band densities from the Western immunoblots. Values are expressed as mean ± standard error. ***$p < 0.001$. *Source: Figure is reprinted from Ref. 491, with permission.*

and acute sexual experience compared to naive males and those that had only chronic or only acute mating experience. Therefore, D_1 receptors in the MPOA, possibly acting through a cAMP/PKA intracellular mechanism, mediate the experience-induced enhancement of copulation.

SEROTONIN

5-HT is generally inhibitory to male sexual behavior, and large doses of 5-HT microinjected into the MPOA did impair copulation[202]; furthermore, a 5-HT_{1B} agonist delayed ejaculation.[202] Reverse dialysis of 5-HT into the MPOA impaired ejaculation and also attenuated glutamate release, which normally increases during copulation, with a 300% rise in the 2-min sample during which the male ejaculated.[492] In accord with the facilitative effects of systemically administered 5-HT_{1A} agonists, 8-OH-DPAT microinjected into the MPOA facilitated ejaculation.[201] Administered by reverse dialysis into the MPOA, 8-OH-DPAT also facilitated copulation[203] and increased both DA and 5-HT levels.[493] The facilitative effects of 8-OH-DPAT were partially blocked by the D_2 antagonist raclopride, but not by the 5-HT_{1A} antagonist p-MPPI, suggesting that the elevation of DA levels, resulting in stimulation of D_2 receptors, mediated much of the facilitation.[203]

GAMMA-AMINO BUTYRIC ACID

GABA is a ubiquitous inhibitory neurotransmitter in adult mammals. As expected, the GABAergic drugs muscimol and ethanolamine-O-sulfate, microinjected into the MPOA, decreased the numbers of male rats that mounted, intromitted, or ejaculated, whereas the $GABA_A$ antagonists bicuculline or 3-mercaptopropionic acid (3-MPA) dramatically shortened ejaculation latency and postejaculatory intervals and decreased the numbers of intromissions preceding ejaculation.[494] However, MPOA administration of the same two antagonists failed to restore copulation in satiated males, again suggesting that the postejaculatory interval is governed by mechanisms separate from those that regulate sexual satiety.

OPIOIDS

As with systemic manipulations, studies using microinjections of opiates or opioids into the MPOA suggest that low doses of agonists facilitate, and high doses inhibit, copulation. The lowest doses of the μ agonist morphine and of the κ agonist dynorphin (1–13) decreased the time and numbers of intromissions before ejaculation, and the highest dose of morphine resulted in failure to resume copulation after the second ejaculation.[495] The selective μ agonist morphiceptin delayed the onset of copulation but had no effect on copulatory performance, touch-based erections, sexual motivation, or locomotion.[496] However, the potent μ agonist β-endorphin microinjected into the MPOA both delayed copulation and inhibited its performance.[497] In support of the importance of low levels of endogenous opioids, naloxone in the MPOA prevented induction of sexual reinforcement.[498]

NOREPINEPHRINE

Administration of NE into the MPOA facilitated both sexual arousal and copulatory performance; conversely, the β-noradrenergic antagonist propranolol and the α-antagonist phenoxybenzamine inhibited copulation.[499] The α_2 agonist clonidine, which would have decreased NE release by stimulating autoreceptors, decreased the number of animals that copulated and increased ejaculation latency and interintromission interval; however, intromissions preceding ejaculation decreased.[500] In addition, the α_2 antagonist yohimbine (which would have disinhibited NE release) in the MPOA blocked the inhibitory effects of systemically injected clonidine.[500] Therefore, stimulation of α_2 receptors in the MPOA appears to facilitate copulation.

NITRIC OXIDE

As with systemic manipulations, local increases in NO facilitate some aspects of copulation. Reverse dialysis of its precursor, L-arginine, into the MPOA increased mount rate, whereas the NOS inhibitor L-NMMA decreased that measure.[501] L-NMMA microinjections increased the number of *ex-copula* seminal emissions and decreased the number of inexperienced males that copulated, although it did not affect experienced males.[502] L-NAME microinjections into the MPOA prevented intromissions and ejaculations in sexually naive males and decreased the numbers of intromissions and ejaculations in experienced males.[503] Furthermore, in naive males, L-NAME microinjections before each of seven exposures to an inaccessible female prevented the facilitative effect of those exposures, which was observed in saline-treated males, compared to non-exposed animals.[503]

Nitrergic function in the MPOA is hormonally regulated. Castration of male rats decreased production of cGMP (the most common second messenger of NO) and blocked NMDA-induced increases in plasma GnRH and in cGMP in MPOA slices.[504] Castration also decreased nNOS-IR in the MPN of male hamsters and rats (reviewed in Ref. 3). T replacement for 2, 5, or 10 days in castrated rats resulted in progressive improvement in copulation and increases in NOS-IR; shorter mount latencies were correlated with greater NOS-IR density. Either E or T replacement, but not DHT, was able to restore NOS-IR.[505] However, in DHT-treated castrates (to maintain peripheral structures) reverse dialysis of an nNOS donor (sodium nitroprusside, SNP) increased DA release and restored ejaculation in half of the animals (Figure 49.15).[506] The lack of full restoration suggests that NO in other brain sites also contributes to mating ability or that unilateral reverse dialysis of an NO donor does not provide a sufficient amount of NO for full restoration. Furthermore, ERα knockout mice had less nNOS-IR than wild-type mice or mice lacking AR because of the testicular feminization mutation (*tfm*).[507] Thus, the aromatization of T to E likely mediates the upregulation of NOS-IR. Gonadal steroid receptors are co-localized with nNOS-IR in mice, rats, and hamsters (reviewed in Ref. 3). In contrast to the castration-induced decreases in NOS-IR in the above studies, there is one report of increased NOS-IR and activity after castration and reduction by either T or DHT.[508] The reason for this apparent discrepancy is not clear, but may be related to the more posterior location examined by Singh and colleagues or their use of an antibody that labeled very few cells. In summary, NO in the MPOA enhances copulation and increases GnRH release. T, probably through aromatization to E and activation of ERα, increases production of NOS in the MPN, although apparently not in more posterior areas.

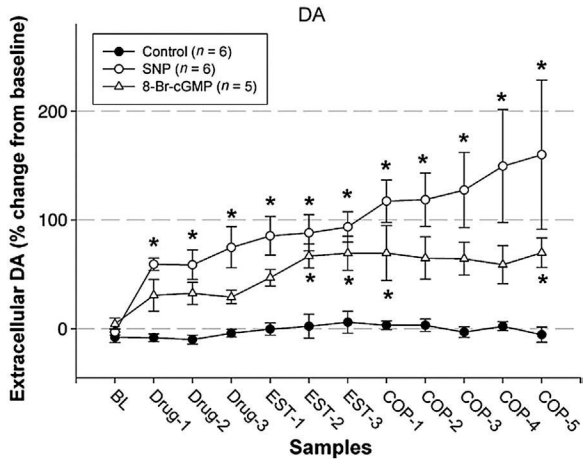

FIGURE 49.15 In DHT-treated castrates (used to maintain peripheral structures), reverse dialysis of an nNOS donor (sodium nitroprusside, SNP) increased dopamine (DA) release and restored ejaculation in half of the animals (not shown). While in 8-Br-cGMP-treated animals, DA levels increased relative to baseline (BL), but reached significance at only the second and the third estrous female samples (EST-2 and EST-3), when the female is presented above the male's cage, as well as at the first and the last copulation samples (COP-1 and COP-5), when males copulated with a sexually receptive female. Values are expressed as mean ± standard error. *$p < 0.05$. *Source: Figure is reprinted from Ref. 506, with permission.*

GLUTAMATE

Microinjection of the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 into the MPOA of sexually naive male rats prevented copulation, and in sexually experienced males, impaired copulation.[509] Furthermore, in sexually naive males microinjection of MK-801 before each of seven noncopulatory exposures to an estrous female prevented the facilitation of such exposures on a drug-free test on day eight that was observed in saline-treated males, compared to unexposed males. Therefore, endogenous glutamatergic stimulation of NMDA receptors in the MPOA facilitates copulation in both naive and experienced males and is permissive for the sensitization to female cues by repeated exposures to a female in sexually naive males. In tests of genital reflexes in anesthetized male rats, microinjection of glutamate into the MPOA elicited erectile responses[456] and the UG reflex.[458]

ACETYLCHOLINE

The nonspecific ACh agonist carbachol or the muscarinic agonist oxotremorine, microinjected into the MPOA, decreased the numbers of intromissions preceding the first ejaculation.[510] This effect was blocked by the muscarinic antagonist scopolamine, which also decreased the numbers of animals that mounted, intromitted, or ejaculated.[510] Acetylcholinesterase immunoreactivity in the SDA was more dense in male gerbils than in females; castration decreased staining intensity, which was restored by T replacement.[510a] Therefore, ACh in the

MPOA may contribute to the hormonal control of sexual behavior.

PROSTAGLANDIN E_2

Bilateral administration of PGE_2 into the MPOA of castrated male rats, maintained on a subthreshold T replacement regimen, activated copulation within 30 min.[511] Half the animals copulated and 40% copulated to ejaculation. Thus, PGE_2 in the MPOA facilitates copulation, possibly by increasing MPOA temperature (see section on systemic manipulation of PGE_2).

OREXIN/HYPOCRETIN

Orexin/hypocretin neurons, located in the perifornical lateral hypothalamus, are better known for their facilitation of feeding[512] and prolonging wakefulness.[512a] However, microinjection of orexin/hypocretin into the MPOA of male rats also facilitated copulation.[513]

OXYTOCIN

Microinjection of oxytocin into the MPOA facilitates male rat sexual behavior, and an antagonist impairs mating.[514] Furthermore, repeated experiences increase both gene expression and protein levels of oxytocin receptors.[515] Therefore, while oxytocin has been studied primarily in the PVN and mesolimbic system, it also works in the MPOA to promote mating.

Electrophysiological Recordings

Electrophysiological recordings have revealed that different neurons in the MPOA participate in the control of sexual motivation and copulatory performance. Some neurons were activated only before male rats began to copulate, while others were activated only during copulation.[516] Similar results were obtained in monkeys, in which neural activity was highest while the animal was making an operant response (pressing a lever) to bring a female closer; activity decreased slightly during copulation and ceased after ejaculation.[517] In slice preparations from the MPOA of Japanese quail, intra- and extracellular recordings revealed that bath applications of DA inhibited most cells (52–80%) but excited a few (10–24%). These effects were not blocked by either D_1 or D_2 antagonists, but were blocked by α_1 or α_2 antagonists, respectively.[518] The effects were not blocked by dopamine–β-hydroxylase inhibitors, indicating that DA was not being converted to NE to produce the effects. Therefore, in quail DA affects neuronal activity via crosstalk with NE receptors.

Chemical Changes Detected by Microdialysis or from Tissue Punches

There is a close association between extracellular MPOA DA levels and sexual behavior in male rats. DA increased as soon as a receptive female was placed behind a perforated barrier; DA levels remained high, or increased further, when the animals were allowed to copulate (Figure 49.16) (reviewed in Ref. 3). The recent presence of T is required for both copulation and the DA response to the female. All gonadally intact males, as well as two-thirds of animals castrated one week before, showed a precopulatory DA increase and copulated when the barrier was removed.[519] The remaining one-third of the one-week castrates, and all two-week castrates, did not show a DA response to the female and did not copulate. There was behavioral specificity, in that neither voluntary exercise in a running wheel nor presentation of another male behind the barrier elicited a DA increase,[519] and eating a palatable food did not increase the DA metabolite DOPAC in a previous study.[520] Furthermore, probes anterior or lateral to the MPOA did not detect a response to the female. The increase in DA before copulation began provides evidence that the DA increase was not caused by copulation, but was more likely associated with sexual motivation.

In the original studies described above, basal levels of extracellular DA were not measured; thus, it was not clear whether basal levels were also affected by

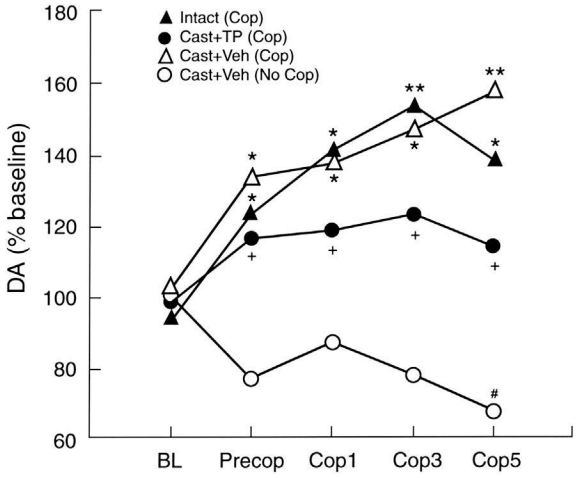

FIGURE 49.16 Extracellular dopamine in the MPOA of male rats during baseline (BL), a precopulatory period (Precop; estrous female behind a perforated barrier), and three 6-min periods after the barrier was removed and the animals were free to copulate (Cop). All gonadally intact males and all castrates treated with testosterone propionate (200 µg/day) showed a significant increase in dopamine during the precopulatory period and during copulation; all of these animals did copulate. A total of nine of 14 oil-treated 1-week castrates also showed the precopulatory dopamine response and copulated after the barrier was removed. The remaining 1-week and all four 2-week oil-treated castrates failed to show the precopulatory dopamine response and failed to copulate; data from these two groups are combined. $^{*}p<0.05$ compared to final baseline for intact males or for 1-week vehicle-treated castrates that copulated. $^{**}p<0.01$ compared to final baseline for intact males or for 1-week vehicle-treated castrates that copulated. $^{+}p<0.05$ compared to baseline for testosterone-treated castrates. $^{\#}p<0.05$ compared to final baseline for vehicle-treated castrates that failed to copulate. *Source: Figure is reprinted from Ref. 519, with permission.*

castration, or only the response to a female. Therefore, the no-net-flux technique was used to measure absolute basal levels in castrates, compared to gonadally intact males.[521] Briefly, different amounts of DA are added to the dialysate; if there is less DA in the brain than in the dialysate, some will diffuse out of the dialysate into the brain, and the loss can be detected.[522] Conversely, if there is less DA in the dialysate, or none, as in standard dialysis, DA will diffuse from the brain into the dialysate, and the gain can be detected. A regression line is drawn, and the point at which it crosses from loss to gain represents the basal extracellular DA level. In castrates the absolute extracellular DA levels were only about one-fifth those of gonadally intact males (0.3 versus 1.3 nM[521]). Therefore, the deficit in castrates was a general one, not limited to the increase in response to a female. However, systemically administered amphetamine elicited greater DA release in castrates than in intact males, and there was more DA in tissue punches from castrates than in those from intact males.[521] Therefore, synthesis and storage of DA were at least as great in castrates as in intact males, but castrates were unable to release their abundant stores.

Further evidence for an association between DA release and the ability of castrates to copulate was provided by a study of restoration of copulation by 2-, 5-, or 10-day regimens of T treatment.[86] No 2-day T-treated castrate showed a DA response to the female and none copulated. Eight of nine 5-day T-treated castrates showed a DA response, and those eight copulated, with five also able to ejaculate. All 10-day T-treated castrates showed a precopulatory DA response, and all copulated. There were numerous correlations between DA release and copulatory measures. Therefore, both loss of copulation following castration and its restoration following T replacement were closely associated with female-elicited DA release in the MPOA.

A major factor regulating DA release in the MPOA is NO. Both basal[523] and copulation-induced[524] DA release were dependent, in part, on NO. Furthermore, NOS-IR in the MPN is positively regulated by T and E[505,507,525–527]; but see Ref. 508. Therefore, T may maintain both basal and female-stimulated DA release by upregulating NOS in the MPOA.

T's metabolites were differentially effective in restoring basal and female-stimulated DA release in long-term castrates.[98] E restored high basal levels of DA, but not the increase in response to a female. E-treated males all intromitted, but none showed a behavioral ejaculation pattern. Neither DHT nor oil vehicle maintained basal DA levels or a female-stimulated increase, and no DHT- or oil-treated animal copulated. Although DHT was ineffective by itself, when it was administered together with E, the combination effectively restored basal and female-stimulated DA responses, as well as copulation.

Although extracellular DA levels were positively correlated with copulatory measures, tissue (stored) DA levels were negatively associated with copulation.[505] The noncopulating groups (DHT and oil) had higher tissue levels of DA than did the groups that copulated (E, E+DHT, and T). As in the Du et al.[521] study above, DHT- and oil-treated animals could synthesize and store DA, but had difficulty releasing it in basal conditions and also in response to a female. Furthermore, a deficiency in NO may have led to the impaired DA release; DHT- and oil-treated males had less NOS-IR than did the three groups that were able to copulate.[505] A similar finding regarding an apparent DA response to stimuli from a female was recently reported regarding juvenile versus adult male hamsters.[528] Adult hamsters showed an increase in DOPAC in punches from the MPOA in response to presentation of vaginal secretions from an estrous female, suggesting release of DA and subsequent uptake and metabolism, whereas juveniles showed no such response and were unable to copulate.

A major factor regulating the MPOA DA response to a female is input from the Me. Small radiofrequency lesions of this area in highly experienced rats increased both the number of intromissions and the time preceding ejaculation and decreased the number of ejaculations[355] (see the section Amygdala above). These lesions did not affect basal DA levels, but they abolished the DA increase in response to an estrous female (Figure 49.17). Thus, as

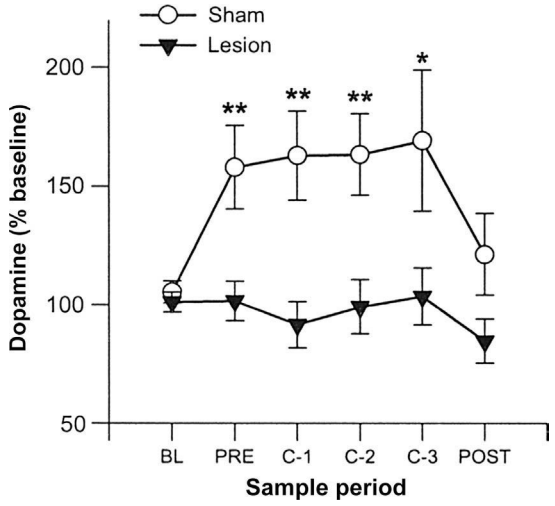

FIGURE 49.17 Levels of dopamine in dialysate from the MPOA of animals with medial amygdala lesions. Levels represent percent changes from baseline (BL) in response to precopulatory exposure to an estrous female (PRE), during copulation (C-1 to C-3), and after copulation (POST). Extracellular levels of dopamine significantly increased during the precopulatory and copulatory stages of testing for animals with sham lesions but not for animals with medial amygdalar lesions. The baseline value used for computation was obtained by dividing the value of the last baseline by the mean of all three baselines. Values are expressed as mean + SEM. *$p < 0.05$; **$p < 0.01$. *Source: Figure is reprinted from Ref. 355, with permission.*

with E2 treatment of castrates,[98] normal basal levels of MPOA DA were compatible with suboptimal copulatory ability. However, an increase in DA before and during copulation appears to contribute to the ability to ejaculate. As noted above, apomorphine microinjected into the MPOA of males with large excitotoxic lesions of the amygdala completely restored their copulatory ability, which had been almost abolished by the lesions.[355] Therefore, input from the Me is important for increasing DA release in the MPOA in response to a female and during copulation, and this is a major way in which the Me facilitates copulatory ability. A recent critical review observed that "there was no delay whatsoever with regard to the initiation of sexual behavior despite the absence of preoptic DA release" in the Dominguez et al.[355] article.[167] They suggested, therefore, that DA has little or nothing to do with sexual motivation. However, the data do show trends that approach statistical significance for both mount latency and intromission latency; the relatively high variability among animals with lesions may have contributed to the lack of statistical significance. Furthermore, there was not an "absence of DA release" but rather a lack of female-stimulated increase in DA; basal DA levels were normal and, as we have suggested, are sufficient for suboptimal copulation. As noted above, DA in the MPOA increased as soon as an estrous female was presented behind a barrier; thus, it was not elicited by copulation per se, and may represent motivation to gain access to the female.[519] In addition, in Japanese quail, as in rats, the presence of a receptive female elicited DA release in the MPOA, but only in males that did copulate (Figure 49.18).[529,530] In males that did copulate, the DA did not fall during the periods between actual mating behaviors, which are only about 4 s long. The authors suggest that the DA release is not simply triggered during actual copulation and occurs only in males that are motivated to copulate. Therefore, the data from both microinjection and microdialysis experiments suggest that MPOA DA does contribute to sexual motivation, copulatory ability, and genital reflexes, although other structures and other neurotransmitters also contribute to these processes.

The olfactory bulbs apparently provide the signal relayed by the amygdala to the MPOA. Male hamsters with sham bulbectomy, or with unilateral bulbectomy contralateral to the MPOA microdialysis site, showed increased extracellular DA in the MPOA when presented with an estrous female.[320] However, bilateral or ipsilateral bulbectomy inhibited the DA response and impaired copulatory behavior, similar to the results of Dominguez et al.[355]

In sexually mature male hamsters, pheromones from estrous females elicited an apparent increase in DA release, inferred from an increase in the DA metabolite DOPAC, in tissue from the MPOA.[528] However, tissue from prepubertal males did not show such an increase.

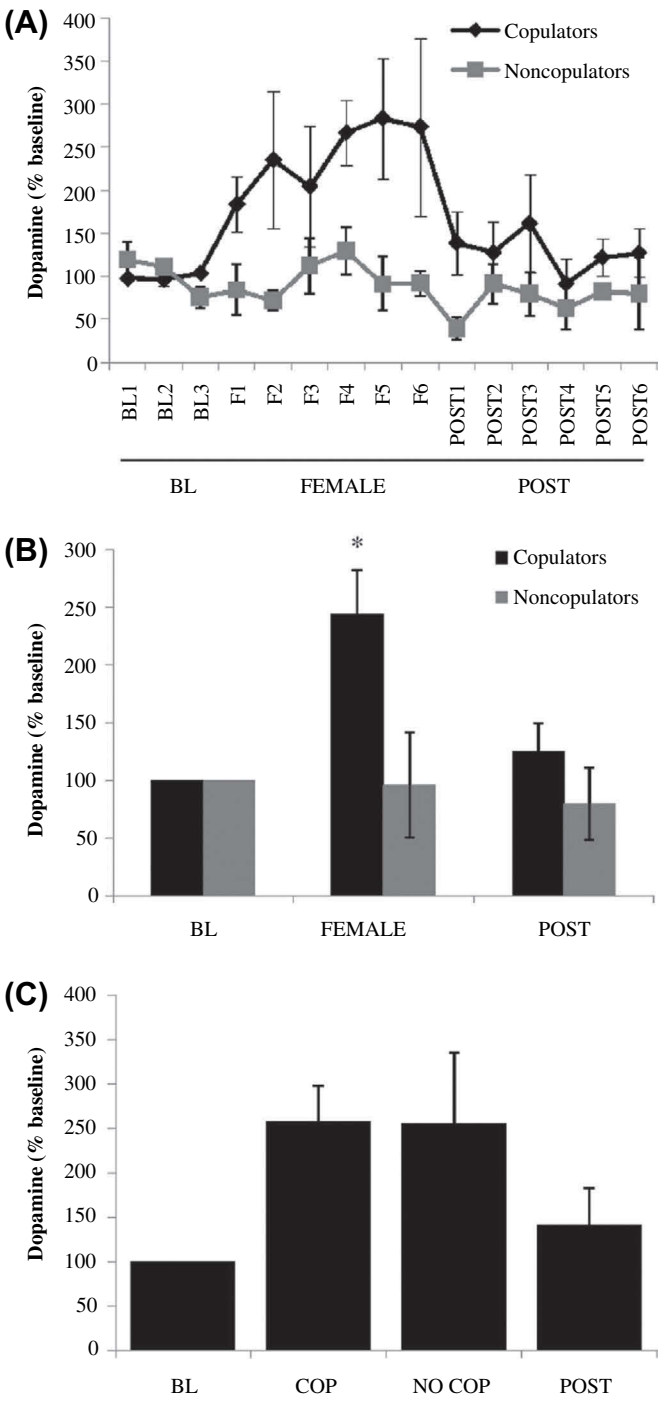

FIGURE 49.18 Extracellular dopamine (DA) in the medial preoptic area (MPOA) of Japanese quail changes in the presence of a female. A and B depict mean change in MPOA DA during baseline (BL), in the presence of a female (FEMALE), and after the female was removed (POST). Panel C depicts mean change in MPOA DA during copulation (COP) and no copulation (NO COP), within the same birds. In males that did copulate, the DA did not fall during the periods between actual mating behaviors. Samples were collected at 6-min intervals. Values are % baseline and expressed as mean ± SEM. *$p < 0.05$. *Source: Figure is reprinted from Ref. 529 with permission.*

Therefore, the neural processing of sexually relevant chemosensory stimuli matures during puberty.

The 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) increased in dialysates from the POA of male rats that had ejaculated,[531] and 5-HT itself was increased in tissue punches from the POA following ejaculation.[532] These authors suggested that postejaculatory 5-HT increases in the POA contributed to postejaculatory quiescence. However, a more recent study, based on analysis of 5-HT itself, concluded that MPOA and, more laterally, POA 5-HT levels were constant throughout copulation and the postejaculatory interval.[533] However, 5-HT was increased in the anterior lateral hypothalamic area (LHA) at the time of ejaculation. The increase in 5-HIAA observed by Refs 534 and 531 may have resulted from diffusion from the adjacent LHA. Therefore, it seems unlikely that 5-HT in the MPOA contributes to postejaculatory quiescence.

Expression of c-fos *and/or Other Immediate–Early Genes*

In keeping with the pivotol role of the MPOA in the control of male sexual behavior, numerous studies in a variety of species have documented Fos induction in the MPOA with sexual activity. Fos is expressed in the MPOA following copulation in male rats, gerbils, mice, and hamsters (reviewed in Ref. 3). In particular, neuronal activation in the MPOA appears to be selective for sexual activity, because mating, but not aggressive behavior, increased Fos-IR in the MPOA of male hamsters.[535] Sexual experience may also prime MPOA neurons to be more responsive to sexual stimuli. The number of Fos-IR neurons in the MPN following one ejaculation was greater in sexually experienced male rats compared to naive males, even though experienced males required fewer intromissions to reach ejaculation.[536] Likewise, chemosensory cues were more effective in stimulating Fos expression in MPOA of sexually experienced male hamsters.[335]

Fos is expressed in androgen receptor-containing neurons of the MPOA in rats,[373] but Fos does not co-localize with aromatase in quail MPOA.[537] Using retrograde tract-tracing, Fos-positive neurons were demonstrated in Me and CTF that project to the MPOA.[373] Heeb and Yahr[538] reported similar findings in male gerbils. Moreover, mating-induced Fos expression in the MPOA requires afferent input from both Me and CTF, because combined ipsilateral lesions of Me and CTF blocked copulation-induced Fos in the MPN, though single lesions did not affect Fos.[337] In turn, Fos-positive neurons in the MPOA send their projections to the PAG.[539]

Fos may be expressed in the MPOA in response to female odors alone, although there is less consensus on this point. Sexually experienced male rats exposed to estrous-female bedding showed an increase in Fos-IR in the MPOA.[340,369] Likewise, chemosensory cues from females activated Fos in the MPOA of male hamsters,[371,540] macaques,[409] gerbils,[374] and aromatase-knockout mice.[382] Similar results were obtained using fMRI in marmosets[541] or with brain temperature recordings in male rats.[542] In addition, electrical stimulation of the vomeronasal organ activated Fos in the MPOA of male hamsters.[543] Finally, exposure to sexual conditioned stimuli increased Fos-IR in the MPOA of male Japanese quail; neural activation was higher in subjects exposed sequentially to the CS and copulation than in subjects exposed to copulation or the CS alone.[13] However, other investigators did not report an MPOA Fos response to female odors in rats,[337,421] mice,[338] hamsters,[333] or ferrets.[344] It may be that certain subregions of the MPOA are activated selectively by ejaculation while others respond preferentially to chemosensory cues. In this regard, chemosensory cues selectively activated the medial and lateral sexually dimorphic areas of the male gerbil,[374] as well as the magnocellular subdivision of the MPOA in hamsters.[371,540] However, ejaculation activated Fos within the medial preoptic nucleus of both hamsters[540] and rats.[421] In a similar manner, the posterodorsal preoptic nucleus (PdPN) in gerbils expressed Fos only after ejaculation.[374] Whether chemosensory cues inducing Fos in MPOA are mediated by the main or accessory olfactory systems remains unclear.[334,335]

The neurochemical identity of Fos-positive neurons has only recently been studied. In male gerbils, approximately half of the Fos-positive cells in the medial sexually dimorphic area and PdPN are GABAergic.[380] Nearly 25% of cells in the medial sexually dimorphic area are glutamatergic, but PdPN neurons do not express glutamate. In male rats, galanin neurons in the MPOA are activated selectively by ejaculation.[380a] Administration of a DA D_1 antagonist before copulation decreased ejaculation-induced Fos,[536] suggesting that at least some of the copulation-induced Fos-IR may result from stimulation of D_1 receptors.

Effects of Intracerebral Grafts

Sexual arousal and copulatory ability decline with advancing age in males of many species. Injection of suspensions of fetal MPOA neurons into the MPOA of 19- to 24-month-old rats improved their copulatory behavior between 21 and 45 days after implantation.[544] The improvements lasted until the end of the experiment 4.5 months later. Grafts from cerebral cortex to MPOA or from MPOA to the ventromedial hypothalamus (VMH) were ineffective. Two hormonal measures, serum T and the postcastration increase in luteinizing hormone, were also increased by the grafts. Therefore, some age-related declines in copulatory ability, sexual motivation, and neuroendocrine function may result at least partially from dysfunction in the MPOA.

Activation of Neurotransmitter Receptors

Endocytosis of μ opioid receptors in the MPOA was observed within 30 min of copulation and was still evident 6 h later.[41] The opioid antagonist naloxone prevented receptor internalization. Microinjections of a μ agonist produced similar endocytosis, and mating resulted in increased Fos-IR in neurons that contained μ receptors. However, naloxone pretreatment did not prevent the Fos response in μ receptor-containing neurons, suggesting that the Fos response did not result directly from μ activation during mating.

Summary of MPOA Functional Roles

The MPOA receives both chemosensory and genitosensory input, as well as information from other sensory modalities. Reciprocal connections with the sources of the sensory input allow hormone-concentrating neurons to bias the sensory input to favor sexually relevant information. Output from the MPOA to other hypothalamic, midbrain, and brainstem nuclei that control somatomotor patterns and genital reflexes contribute to the initiation and orchestration of complex, interactive copulatory behaviors.

Mesocorticolimbic DA Tract

Cell bodies in the ventral tegmental area (VTA) give rise to axons of the mesocorticolimbic DA tract that innervate the nucleus accumbens (Acb), prefrontal cortex, and ventral forebrain areas.[545] This system is critical for appetitive behavior and reinforcement and is activated before and/or during a variety of motivated behaviors, including eating, drinking, sexual activity, drug self-administration, and intracranial self-stimulation.[546,547] It is not clear whether this system is more important for reward processes,[548] behavioral activation,[549–551] "wanting" (as opposed to "liking"[552]), or incentive learning.[553,554] However, it is clearly essential for appetitive behavior. The mesocorticolimbic tract is activated both before and during copulation in male rats. DA released in the Acb is important for the activation of numerous motivated behaviors, including sexual behavior, but appears to have little influence on specifically sexual motivation or on copulation per se. Because most research on this system has focused on the Acb and VTA, this review will also concentrate on these areas.

Effects of Electrolytic or Cell Body Lesions or of Electrical Stimulation

Few recent studies have tested the effects of lesions of the Acb or VTA on sexual behavior. In general, elements of the mesocorticolimbic DA tract appear to be important for sexual arousal in response to remote cues from females, but play a relatively less critical role in copulatory performance per se. Lesions of the VTA and the adjacent interpeduncular nucleus increased the postejaculatory interval, but did not affect other copulatory measures.[555] Likewise, male rats with DA-depleting lesions of the Acb had a lower incidence of NCE, fewer erections, and longer latency to display erection; radiofrequency lesions of the Acb also increased the latency to the first NCE.[556] Nonetheless, copulation was normal in both groups of lesioned rats. Using excitotoxic lesions of the Acb, Kippin et al.[557] observed a reduction in NCE, as well as intromission and ejaculation. However, lesioned male rats maintained a normal preference for receptive females.

Electrical stimulation of the VTA decreased the latencies to mount, intromit, and ejaculate[558,559] and increased the number of ejaculations in a 30-min test.[559] This facilitation was specific to the Acb, as stimulation of the caudate putamen inhibited sexual behavior.[560] Stimulation of the Acb did not reverse sexual satiety.[560] These results are consistent with a general activating effect of the mesocorticolimbic tract.

Effects of Castration or of Direct Applications of Hormones

Although the Acb and VTA are logical sites for gonadal steroid hormones to stimulate sexual motivation, there are relatively few data to support this hypothesis. Intracerebral implants of T into the VTA of castrated male house mice did not restore mounting, ultrasonic vocalizations, urine marking, or attraction to female urine.[561] However, the combined application of T to the MPOA and VTA was more effective than implants in the MPOA alone. T may also be reinforcing, independent of its effects on sexual motivation. Male rats formed a conditioned place preference for intra-Acb injections of T,[562] and T-induced conditioned place preference was blocked by intra-accumbens injections of a DA antagonist.[563] This effect appears to be due to androgenic metabolites of T acting on the shell of the Acb, because intracerebral implants of DHT into the Acb shell also induced conditioned place preference.[564]

The mechanisms through which steroid hormones may act on the mesolimbic DA system are unclear. There are contradictory reports on the effects of castration on Acb DA levels. In male rats, castration depressed DA concentrations in the Acb, and this effect was reversed by replacement with T, E2, or DHT.[565,566] However, Hernandez et al.[567] found that amphetamine released more DA in dialysates from prepubertally castrated males, suggesting that there may be more DA stored in tissue. Likewise, the presence of steroid receptor-containing neurons in the Acb or VTA remains controversial. Early studies reported no binding of hormones to classic steroid receptors in the VTA, though low levels of binding were found in the Acb.[568,569] A more recent study found

small patches of AR neurons in the VTA that were also immunoreactive for tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis.[570] Despite the scarcity of AR and ER in the mesolimbic DA system, there is the additional possibility that gonadal steroids may act on Acb and VTA via nongenomic receptors.

Expression of c-fos *or Other Immediate–Early Genes or Receptor Activation*

Mating stimulated Fos in the Acb of male rats.[337,339] Moreover, sexual experience enhanced Fos-IR in the Acb induced by an estrous female, particularly in the Acb shell.[12] Fos was expressed in the Acb in response to female odors, but was also conditioned to a nonsexual odor paired with mating.[354] Conditioned odor cues also stimulated Fos-IR in the VTA.[571] Increased Fos-IR in the VTA of male rats that were exposed to sex-related cues or that copulated was found in both dopaminergic (tyrosine hydroxylase-IR) and nondopaminergic neurons.[572] Both types of stimuli also activated neurons in the Acb core and shell. In addition, μ opioid receptors in the VTA were internalized following both sex-related cues and copulation. The authors suggested that μ receptors on GABAergic neurons in the VTA release dopaminergic neurons from tonic inhibition. Tail pinch, an arousing stimulus that can promote copulation in olfactory bulbectomized males[326] or castrates maintained on subnormal T replacement[229] (reviewed above), also increased Fos-IR in the VTA as well as the MPOA, BST, paraventricular nucleus (PVN), Me, and several sites not usually associated with copulation. However, Fos expression in the Acb in response to sexual activity may be limited to rats. In hamsters and gerbils, no mating-induced increase in Acb Fos-IR was observed.[374,379]

Effects of Direct Applications of Drugs Affecting Specific Transmitters

DOPAMINE

Stimulation of impulse-regulating autoreceptors in the VTA with apomorphine delayed the onset of copulation and slowed its rate.[573] Similar microinjections slowed the speed of running in all four arms of an X-maze and increased the number of trials on which the male failed to leave the start area; however, neither the percentage of trials on which the male chose the female's goal box nor *ex-copula* genital reflexes were altered.[574] Thus, inhibiting mesocorticolimbic DA activity decreased behavioral activation but had no effect on specifically sexual motivation or on genital reflexes.

Application of drugs to the Acb also affected primarily activity measures. The onset of copulation was speeded by amphetamine and delayed by *cis*-flupenthixol or the DA neurotoxin 6-OHDA (reviewed in Ref. 353). Amphetamine also increased responding for a secondary reinforcer that had been paired with copulation.[353] The D_2/D_3 agonist quinelorane increased the number of trials on which the male failed to leave the start area but did not affect the percentage of trials on which he chose the female's goal box or his copulatory behavior when he reached the female.[490] It is not clear whether quinelorane's effects resulted from stimulation of terminal autoreceptors or postsynaptic receptors. However, drug injections into either the VTA or the Acb have affected general activation, with little influence on specifically sexual motivation or on copulatory measures.

OTHER NEUROTRANSMITTERS

A cholecystokinin A (CCK_A) antagonist, microinjected into the posteromedian Acb, attenuated the facilitation that resulted from electrical stimulation of the VTA, as did either a CCK_A or CCK_B antagonist into the anterolateral Acb.[559] CCK is located in dopaminergic terminals in the posteromedian Acb, but is in nondopaminergic terminals in the anterolateral portion.[575] These data suggest that CCK potentiates the facilitative effects of mesocorticolimbic DA activity.

5-HT microinjected into the Acb inhibited ejaculation (increased mounts, intromissions, and time preceding ejaculation); the 5-HT_{1A} agonist 8-OH-DPAT decreased those same measures.[576] Injection of these compounds into the dorsal striatum was without effect. The authors suggested that 8-OH-DPAT's effects may have resulted from antagonism of 5-HT_2 receptors.

Microinjection of the opioid antagonist naloxone into the VTA decreased anticipatory level changing in a bilevel apparatus but had no effect on sexual performance; β-endorphin had no effect.[233] Microinjections of the neuropeptide oxytocin into the VTA induced penile erections; this effect was inhibited by administration of an oxytocin receptor antagonist or DA receptor antagonist.[577] In addition, oxytocin-induced erections correlated with increased dopaminergic activity in the Acb and PVN.[577]

Chemical Changes Detected by Microdialysis or Voltammetry

Exposure of a male rat to the odor of an estrous female, but not of a nonestrous female, increased extracellular DA in the Acb.[534,578–580] DA levels also increased during copulation.[534,580,581] The DA response to the female's odor occurred on the first exposure to the odor[580] and was attenuated by systemically injected naloxone, which may have blocked the disinhibition of DA neurons by endogenous opioids.[582]

Finer temporal analysis, using in vivo voltammetry[583,584] or microdialysis with capillary chromatography,[585] revealed that Acb DA rose in response to the presentation of the female, increased further during

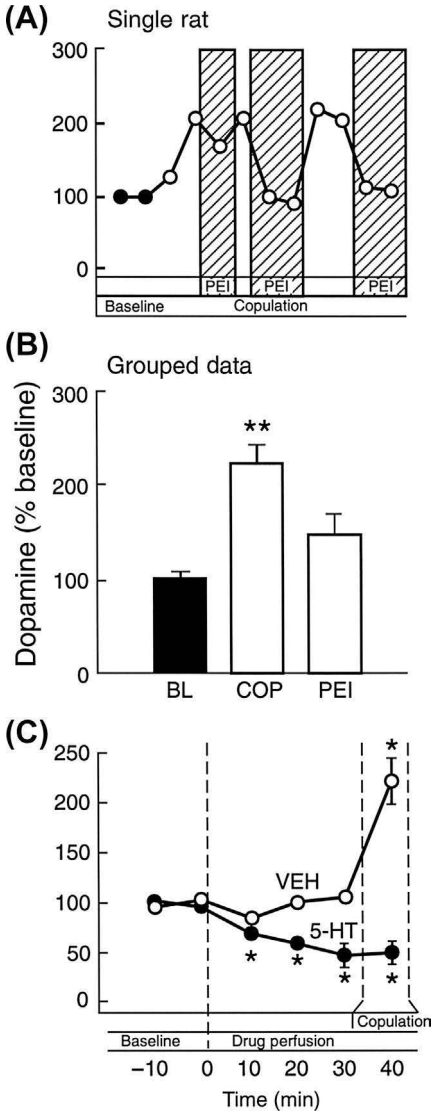

FIGURE 49.19 Levels of dopamine in microdialysate collected from the nucleus accumbens (Acb) of male rats during sexual behavior sessions. (A) Representative example of temporal changes in a single rat. (B) Mean change in DA levels (+SE) for a group of six rats. Samples were collected at 3-min intervals during three ejaculatory series and are designated precopulatory baseline (BL), copulation (COP) or postejaculatory interval (PEI). Dopamine content was significantly greater in samples collected during copulation, compared with samples collected under baseline and postejaculatory conditions (**$p < 0.01$). (C) Temporal change in dopamine concentration of the dialysates collected from the Acb before and during 40 min of 5-HT perfusion into the LHA of six male rats. Samples were collected at 10-min intervals. An estrous female was introduced to the male, and copulation was allowed during collection of the final sample during 5-HT perfusion. A significant decrease in dopamine occurred throughout the entire 5-HT perfusion period. This treatment blocked the increase in Acb DA seen in control animals during copulation.*$p < 0.05$, perfusion times versus time 0 (baseline). *Source: Figure is from Ref. 585, with permission.*

copulation, fell following each ejaculation, and increased again when the male resumed copulation (Figure 49.19). Reverse dialysis of 5-HT into the LHA decreased basal DA in the Acb and prevented the DA response to the female.[585] Because 5-HT is increased in the LHA with ejaculation,[533] one factor contributing to the postejaculatory interval may be decreased Acb DA, as a result of 5-HT release in the LHA.

Sexually satiated male rats, presented with a novel estrous female behind a barrier, showed a slight increase in extracellular DA in the Acb; DA rose significantly when the barrier was removed and the animals copulated.[586] Furthermore, sensitization to the motor-activating effects of amphetamine, following repeated systemic injections of amphetamine, also increased the DA response to a female.[147] Therefore, there is cross-sensitization between a psychostimulant drug and a natural motivated behavior. On the other hand, prenatal stress decreased the number of rats that were able to copulate, but did not affect the DA response to an inaccessible female or DA levels during copulation.[587]

Nigrostriatal DA Tract

The nigrostriatal tract arises from the substantia nigra of the midbrain (A9 cell group) and innervates the dorsal striatum (caudate putamen). Nigrostriatal activity enhances the readiness to respond to external stimuli.[588] Loss of nigrostriatal neurons in Parkinson's disease leads to difficulty in initiating movements and to slowing of movements. Nigrostriatal neurons respond rapidly to any sudden stimulus, but do not contribute to specific information about those stimuli.[589]

Effects of Electrolytic or Cell Body Lesions

Bilateral lesions of the substantia nigra slowed copulation and decreased the number of ejaculations.[555] These data support the proposal that nigrostriatal activity contributes to the somatomotor patterns of copulation.

Chemical Changes Detected by Microdialysis or Voltammetry

DA was released in the dorsal striatum only during copulation, in contrast to the DA release in the Acb and MPOA.[578] Therefore, nigrostriatal DA appears to be more important for motor activation than for sexual motivation.

Expression of c-fos *and/or Other Immediate–Early Genes*

Copulation elicited no increase in Fos-IR in the dorsal striatum, in contrast to the significant increases in the Acb, MPOA, BST, and piriform cortex.[339]

Paraventricular Nucleus of the Hypothalamus

The paraventricular nucleus (PVN) of the hypothalamus consists of a magnocellular division, which releases

oxytocin and vasopressin from the posterior pituitary, and a parvocellular division, which projects to several brain areas and the spinal cord (reviewed in Ref. 590). Axons projecting to the spinal cord contain oxytocin, vasopressin, somatostatin, DA, or other, undetermined neurotransmitters. The parvocellular division receives monoaminergic input from noradrenergic and serotonergic brainstem nuclei, as well as from periventricular DA neurons. The PVN is an important integrative site for autonomic and neuroendocrine functions. It is important for both NCE and seminal emission, but provides less critical contributions to touch-based erections and copulation. Oxytocinergic neurons projecting to several areas of the brain and spinal cord mediate much of the PVN's influence, and DA, glutamate, hexarelin analog peptides, and NO provide excitatory drive to those oxytocinergic efferents. GABA and opioid peptides and opiate drugs inhibit these neurons.

Effects of Electrolytic or Cell Body Lesions

Excitotoxic lesions of the parvocellular PVN decreased NCE but did not affect copulation.[591] Similar parvocellular lesions also failed to affect copulation but did decrease the amount of semen ejaculated and also decreased the number of oxytocin IR fibers in the lumbosacral spinal cord.[592] Larger lesions encompassing both the parvo- and magnocellular divisions inhibited both noncontact and touch-based erections and, in copulation tests, decreased intromission ratio and increased ejaculation latency.[591] On the other hand, electrolytic lesions of the PVN only decreased the latency to the first touch-based erection (i.e., facilitated erection).[197] Therefore, the PVN appears to contribute to both NCE and seminal emission, but PVN lesions have inconsistent effects on touch-based erections and copulation.

Lesions of the lateral parvocellular portion of the PVN destroyed the neurophysin-containing axons to the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB).[593] Neurophysin is used as a marker for oxytocin and vasopressin. Neurophysin-containing terminals appeared to be in contact with somas and dendrites of the SNB. Thus, PVN is apparently the source of oxytocinergic neurons to the SNB, which promote seminal emission and ejaculation.

Effects of Direct Applications of Drugs Affecting Specific Transmitters

DOPAMINE AND OXYTOCIN

Microinjections of the DA agonist apomorphine,[594] the D_2 agonist quinpirole, or oxytocin[595] into the PVN elicited drug-induced erections, which were blocked by an oxytocin antagonist microinjected ICV.[596] PVN microinjections of apomorphine[479] or the D_2/D_3 agonist quinelorane[597] also increased touch-based erections and *ex-copula* seminal emissions. In addition, apomorphine in the PVN elicited increases in intracavernous pressure in anesthetized rats, a response that was enhanced by systemic administration of the monoamine oxidase B inhibitor, selegiline.[598] An oxytocin antagonist administered ICV, but not into the PVN, inhibited NCE,[599] suggesting that axons from the PVN, terminating perhaps in the hippocampus,[600] facilitate NCE. A more recent

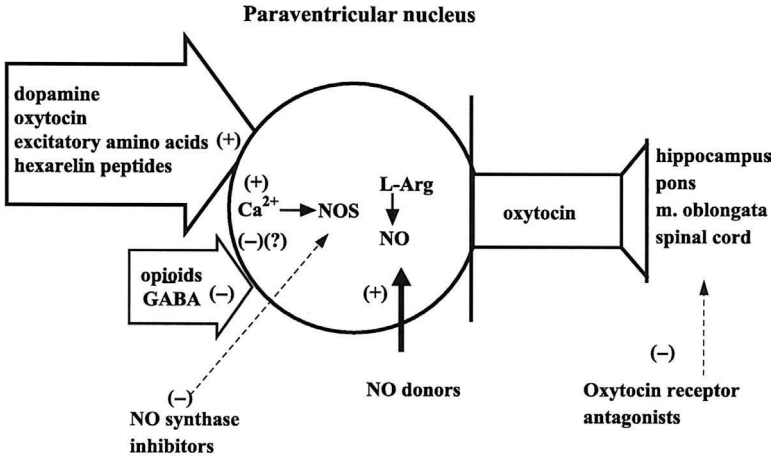

FIGURE 49.20 A schematic representation of oxytocinergic neurons originating in the paraventricular nucleus (PVN) of the hypothalamus and projecting to extrahypothalamic brain areas and the spinal cord involved in sexual activity. Activation of these neurons by dopamine, excitatory amino acids, oxytocin itself, and hexarelin analog peptides facilitates penile erection and sexual activity. The direct or indirect activation of these neurons and the facilitative effects on erection and sexual activity induced by the above substances can be reduced or abolished by the stimulation of opioid and GABAergic receptors. These oxytocinergic neurons are activated by the activation of NOS, present in their cell bodies. Endogenous NO, formed by stimulation of dopamine, excitatory amino acid, or oxytocin receptors; or exogenous NO, derived from NO donors delivered to the PVN, activates oxytocinergic neurons by a yet-unidentified mechanism. This causes the release of oxytocin in brain areas distant from the PVN, as well as the spinal cord, inducing penile erection. Similar mechanisms operate during noncontact erections and copulation. *Source: Figure reproduced from Ref. 602, with permission.*

article suggests that stimulation of D2 receptors in the PVN increases Ca^{2+} influx into oxytocin neurons, leading to production of NO and the resultant release of oxytocin in extra-hypothalamic sites and spinal cord, which increases penile erection and yawning.[601] In summary, DA, acting via D_2 receptors, apparently increases oxytocin release, which in turn promotes drug-induced and NCE (Figure 49.20) (reviewed in Refs 602,603).

NITRIC OXIDE

PVN administration of the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) decreased NCE and impaired copulation in male rats.[604] Another NOS inhibitor, N(G)-monomethyl L-arginine (L-NMMA), reverse-dialyzed into the PVN, decreased touch-based erections, whereas the NO precursor L-arginine increased erections; however, copulation was not affected by either drug.[605] These authors noted that similar administration of L-NMMA into the MPOA did decrease the rate of mounting in copulation tests.[501] Therefore, manipulations of NO in the PVN have had inconsistent effects on copulation, but NO antagonists have consistently inhibited noncontact and touch-based erections.

AMINO ACIDS

The glutamate agonist N-methyl-D-aspartate (NMDA), microinjected into the PVN, elicited erections; this effect was blocked by the NMDA antagonist MK-801 (dizocilpine) and by the NOS antagonist L-NAME.[606] Therefore, glutamate, by opening calcium channels associated with NMDA receptors, may lead to calcium activation of calcium-calmodulin kinase (CAM kinase), which in turn activates NOS. Conversely, microinjections of the $GABA_A$ agonist muscimol into the PVN inhibited erections elicited by apomorphine, oxytocin, or NMDA.[607] The $GABA_B$ agonist baclofen was ineffective. Extracellular levels of glutamate and aspartic acid in the PVN increased during exposure to a receptive female and increased further during copulation.[608] Furthermore, microinjection of an NMDA receptor antagonist (MK-801) into the PVN inhibited both NCE and copulation; an AMPA receptor antagonist (CNQX) also impaired copulation, but to a lesser extent.[608]

ADRENOCORTICOTROPIC HORMONE

Microinjected into the periventricular hypothalamus, including the PVN, ACTH elicited erections.[281] Similar injections into the POA, caudate nucleus, or hippocampus were ineffective.

OPIATES

Morphine, administered unilaterally into the PVN before introduction of an inaccessible estrous female, prevented NCE, as well as the increases in production of NO (see the section Chemical Changes Detected by Microdialysis below) observed with control injections.[609]

HEXARELIN ANALOG PEPTIDES

Hexarelin analog peptides, microinjected into the PVN, elicited erections, probably by stimulating specific receptors on oxytocinergic cell bodies, which increase Ca^{2+} influx and activate NOS (reviewed in Ref. 602).

Chemical Changes Detected by Microdialysis

An increase in NO, inferred from increases in NO_2 and NO_3, was detected in microdialysate from the PVN during both NCE and copulation.[604] This increase was blocked by ICV injection of hemoglobin, an NO scavenger, but not by injections into the PVN; PVN injections of hemoglobin also failed to inhibit NCE.[604] Therefore, NO appears to work intracellularly in the PVN, but to have additional effects in other areas that are important for erections. PVN microinjections of morphine inhibited both copulation and the copulation-induced increase in NO.[609] L-arginine, reverse-dialyzed into the PVN, increased both NO production and touch-based erections, and L-NMMA had the opposite effects.[605] Both NO production and NCE were increased by apomorphine or the D_2 agonist LY-171555, but not by the D_1 agonist SKF-38393.[610] The increase in NO production was blocked by a D_2 antagonist (L-sulpiride) but not by a D_1 antagonist (SCH-23390). However, the increase in erections was blocked by both antagonists, suggesting that both D_1 and D_2 receptors contribute to erectile function. On the other hand, a later study failed to find an inhibitory effect of a D_1 or D_2 antagonist, or of an oxytocin antagonist, administered into the PVN, on NCE or on the production of NO in the PVN, although an NMDA antagonist reduced both.[611] Furthermore, the NMDA microinjections that elicited erections (see above) also increased NO in the PVN.[606]

Microinjection of omega-conotoxin, a potent inhibitor of N-type voltage-activated calcium channels, prevented apomorphine- and oxytocin-induced erections and NO production.[612] Exogenous provision of NO by the NO donors sodium nitroprusside or hydroxylamine overcame the effects of the omega-conotoxin, bypassing the need for activation of N-type channels that were indirectly activated by apomorphine and oxytocin. Omega-conotoxin also failed to block NMDA facilitation of NO production and erections, because NMDA stimulates ligand-activated calcium channels, not voltage-gated channels. Consistent with the facilitative effects of the DA agonist apomorphine and of D_2 agonists, increases in DA release in the PVN were observed during NCE and, to an even greater extent, with copulation.[613]

Presence of Steroid Receptors

The lateral parvocellular subdivision of the PVN projects to the L5-L6 region of the spinal cord; 30% of

those neurons concentrated E2, and almost half of the E2-concentrating neurons projected to the lumbar spinal cord.[614] Therefore, some of the effects of steroids on the SNB may be indirect, mediated by steroid-sensitive afferents.

Expression of c-fos *and/or Other Immediate–Early Genes*

Intromission and ejaculation increased Fos-IR in the parvocellular regions of the PVN, with ejaculation eliciting 2.5 times as many Fos-IR neurons.[615] Ejaculation also elicited more Fos-IR neurons in the magnocellular division than did intromission, although overall numbers were lower than in the parvocellular regions. Half the Fos-IR neurons contained oxytocin, even in noncopulating males. Indeed, copulation did not affect the percentage of double-labeled cells, except in the caudalmost PVN, where one-third of the lateral parvocellular neurons were double-labeled following ejaculation, but no cells were double-labeled in noncopulating animals. However, use of a more selective antibody for Fos, as opposed to Fos plus Fos-related antigens (Fras), revealed no double labeling of Fos and oxytocin following ejaculation in male rats (L. Coolen, personal communication). In gerbils neither exposure to an arena previously associated with copulation, nor copulation itself, increased PVN Fos expression.[374] On the other hand, in a more recent report, copulation did increase the number of neurons double-labeled for oxytocin and Fos-IR.[616] Exposure to an anesthetized female also increased Fos-IR in oxytocin-containing cells in sexually experienced males, but not in naive males. Perhaps variability in Fos-IR in non-oxytocin cells contributed to a lack of statistical significance in the earlier studies that found no Fos increases.

Expression of mRNA or Immunocytochemistry for Neuropeptides

Sexually impotent male rats had less expression of oxytocin mRNA in the PVN, and greater expression of mRNA for proenkephalin and prodynorphin, than did sexually competent males.[617] This finding fits well with the observation that morphine in the PVN inhibited NO production and copulation.[609] In addition, NOS is co-localized with oxytocin in the PVN.[618] Activation of these neurons facilitates *ex-copula* erections and may also contribute to copulation. The oxytocin is released in the hippocampus, where it promotes erections,[600] and in the spinal cord, where it promotes seminal emission.[592]

Efferent Projections

Axons project from the PVN to the nucleus paragigantocellularis (nPGi),[619] a major source of inhibition of genital reflexes and copulation (see the section Nucleus Paragigantocellularis of the Medulla later in the chapter). Axon terminals formed close appositions to serotonergic neurons that projected to the lumbosacral spinal cord and indirectly to the corpus cavernosum. Thus, the PVN is positioned to exert control over inhibitory serotonergic projections to the spinal areas that control erection.

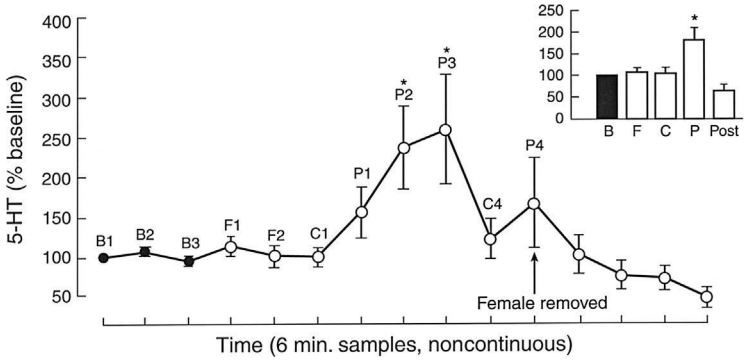

FIGURE 49.21 Temporal changes in extracellular serotonin (5-HT) collected from the lateral hypothalamic area (LHA) of male rats before and during copulation. Each data point is the mean (± SE) for 6-min dialysate samples collected during baseline (B), in the presence of an estrous female (F), during copulation (C), during the postejaculatory interval (P), and after the female was removed (expressed as a % of mean baseline levels). A total of four samples were analyzed after the female was removed, at 30-min intervals. 5-HT levels increased during the second (P2) and third (P3) postejaculatory intervals, compared to final baseline (B3), female behind barrier (F2), and first copulation period (C1). 5-HT during P3 was also higher than during the fourth copulation interval (C4). Samples collected during the second and third copulation series were not analyzed, because most males ejaculated before a full 6-min sample could be collected. The summary graph (inset) represents the mean (± SE) for data from the 15 sample periods collapsed into five groups, based on behavioral condition. Samples collected during the postejaculatory intervals showed higher 5-HT levels compared to all other conditions. Basal extracellular concentrations of 5-HT in the LHA were calculated to be 1.6 ± 0.1 nM. *Source: Figure from Ref. 533, with permission.*

Lateral Hypothalamus

Reciprocal connections of the MPOA with downstream structures pass through the lateral hypothalamus (LH) as part of the medial forebrain bundle,[425] and LH lesions that sever these connections are as effective as MPOA lesions in abolishing copulation. However, LH cell bodies may also influence copulation.

Effects of Direct Applications of Drugs

Microinjection into the anterior LH (LHA) of a selective 5-HT reuptake inhibitor (SSRI) delayed the onset of copulation and delayed ejaculation after the animals did begin to copulate.[533] Thus, the impairment by SSRIs of sexual motivation and ejaculation (reviewed in Ref. 186) may be mediated, in part, by increased 5-HT in the LH.

Changes Detected by Microdialysis

5-HT is released in the LHA at the time of ejaculation, and an SSRI microinjected into the LHA delayed the onset of copulation and delayed ejaculation after the male did begin to copulate (see Figure 49.21).[533] Reverse dialysis of 5-HT into the LHA decreased basal DA levels in the Acb and prevented the usual DA increase during copulation.[585] Because DA release in the Acb contributes to numerous motivated behaviors, the inhibition induced by 5-HT in the LHA may at least partially explain the diminished sexual motivation associated with clinical use of SSRI antidepressants (reviewed in Ref. 186).

Orexin/Hypocretin Neurons

Exposure to a receptive female or copulation increased the number of orexin/hypocretin (orx/hcrt) neurons, located in the perifornical lateral hypothalamus, that were Fos-IR, compared to exposure to another male or placement into an empty cage, respectively.[620] 5-HT inhibits orx/hcrt neurons, which would otherwise excite VTA mesolimbic DA neurons (Figure 49.22).[620] Orx/hcrt neurons are decreased in number by castration and restored by estradiol.[620] Therefore, one means by which SSRIs inhibit mating is via inhibition of orx/hcrt neurons in the LHA that would otherwise activate the mesolimbic system. However, another study reported that specific lesions of orexin/hypocretin neurons did not affect copulation in experienced males and actually shortened the latency to mount in naive males; they also decreased anxiety-like behavior in an elevated plus maze, suggesting that the shortened latencies may have been due to reduced anxiety in the naive males.[621] The reason for the apparent discrepancy between the two studies is not clear.

Ventromedial Hypothalamus

The ventromedial hypothalamus (VMH) is recognized primarily for its role in the control of female rat sexual behavior. (see Chapter 50.) However, the VMH may also modify male sexual behavior. Like the MPOA, the VMH has abundant receptors for androgens and estrogens[411,622] and receives genitosensory input through midbrain projections,[417] as well as chemosensory stimuli via projections from the Me.[402,623,624]

Effects of Electrolytic or Cell Body Lesions

Lesions of VMH often enhance aspects of male sexual activity, suggesting that VMH may tonically inhibit masculine sexual behavior. In this regard, small electrolytic lesions of the VMH in castrated male rats treated with exogenous T reduced the latency to express copulatory behavior.[625] However, in male hamsters, VMH lesions increased ultrasonic vocalizations, but did not alter copulation.[626]

Effects of Hormonal Manipulations

Both ARs and ERs are present in the VMH of male rats[411] and hamsters.[622] Nonetheless, intracerebral implants of T into the VMH in castrated male rats or mice failed to stimulate copulation or ultrasonic vocalizations.[627,628] However, T in the VMH restored partner preference in male rats,[627] and increased urine marking in male mice,[628] suggesting that the VMH contributes to hormone-sensitive

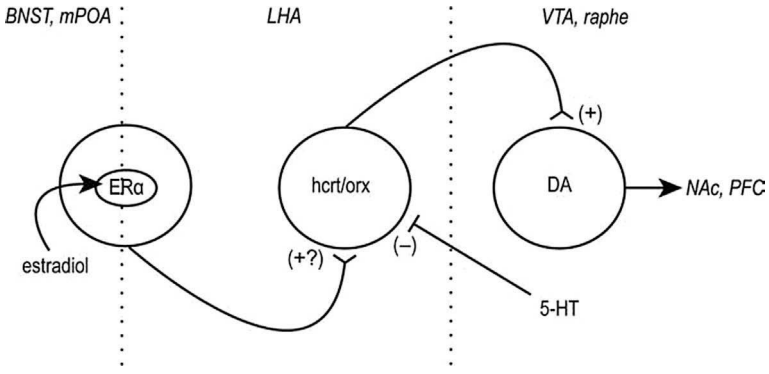

FIGURE 49.22 A model for regulation of hypocretin/orexin (hcrt/orx) by gonadal steroids and ventral tegmental area (VTA) dopamine (DA). Estradiol, synthesized from gonadal testosterone by aromatase, acts on ERα-containing neurons in the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA), and lateral hypothalamic area (LHA). These structures project to hypothalamic hcrt/orx neurons. Excitatory projections from these structures may influence hcrt/orx neuronal and gene expression activity in a steroid-sensitive manner. Hcrt/orx projections to VTA enhance midbrain DA neuronal activity during male sexual behavior. This effect may be blocked by intra-LHA infusions of 5-HT that inhibit hcrt/orx activity, impairing sexual behavior and NAc DA release. *Source: Figure is reprinted from Ref. 620, with permission.*

sexual motivation. Conversely, intracerebral implants of the AR antagonist hydroxyflutamide into the VMH of castrated male rats with T replacement blocked both sexual motivation and sexual behavior.[474] Therefore, it appears that the VMH transduces androgen stimuli important for sexual motivation and copulation. The inhibitory effects of hydroxyflutamide in the VMH stand in contrast to the facilitative effects of VMH lesions described above. It may be that AR-containing neurons inhibit other VMH neurons, or may themselves be inhibited during mating, thereby facilitating male copulatory behavior. Androgen-responsive neurons in the VMH may also affect sexual activity through projections to other sites, including the midbrain.[629]

Expression of c-fos *or Other Immediate–Early Genes*

Mating induced Fos-IR in the VMH in male rats[630] and gerbils.[374] In rats, bedding from estrous females failed to increase Fos-IR in the VMH, suggesting that mating is required to activate Fos expression.[369] However, Fos expression was unaffected by pelvic nerve hemisection in gonadectomized male rats primed with E and progesterone.[631] This suggests that Fos expression in the VMH of males is more dependent on chemosensory input or genitosensory input carried through other pathways, such as the pudendal nerve. By contrast, copulatory activity was not required for the increase in Fos expression in male gerbils.[374]

In contrast to the mating-induced Fos induction observed in the VMH of male rats and gerbils, mating may inhibit neural activity in the VMH of other species. Mating did not increase Fos-IR in the VMH of male musk shrews, although Fos-IR was significantly increased in the VMH of females.[632] Similar results were observed in male hamsters,[540] mice,[338] and ferrets.[344] In male macaques, mating actually elicited less Fos-IR in the VMH than did exposure to an inaccessible female.[409] or exposure to an arena previously associated with copulation.[374]

Midbrain Periaqueductal Gray

In the context of sexual behavior, the midbrain periaqueductal gray (PAG) is primarily associated with vocalization and with lordosis in females. Nonetheless, the PAG has steroid receptors[633] and extensive reciprocal connections with the MPOA (Ref. 424; reviewed in Ref. 3).

Effects of Electrolytic or Cell Body Lesions

The effects of PAG lesions in male rodents are similar to the effects of VMH lesions. That is, lesions of the PAG stimulate sexual activity in male rats.[555] Electrical stimulation of caudal PAG in male guinea pigs produced vocalizations related to sexual behavior.[634] However, combined unilateral lesions of the ventrolateral PAG and the contralateral sexually dimorphic area of the MPOA in male gerbils did not affect mating.[635]

Steroid Receptors and Connections

The caudal two-thirds of the PAG contains a large population of ERα and AR receptors.[636] In the middle third of the PAG, steroid-sensitive neurons were distributed primarily in the dorsomedial and lateral PAG; in caudal PAG, immunoreactive cells were located primarily in the dorsal half. Afferents from the MPOA terminated in close apposition to ERα-IR and AR-IR neurons in the PAG, and between 17% and 54% of PAG neurons that projected to the nucleus paragigantocellularis (nPGi; see the following section) were also immunoreactive for ERα or AR.[633] These data suggest the existence of a hormone-sensitive output pathway for male sexual behavior that extends from the MPOA through the PAG to the nPGi. Similarly, in rhesus monkeys, the PAG projects to the nucleus retroambiguous, a premotor area in the medulla.[637]

MAJOR MOTOR OUTPUTS

Ventral Premammillary Nucleus

The ventral premammillary nucleus receives input from the MPOA, the VMH, and the posterodorsal part of the Me.[623] ARs are located in this nucleus in rats[411] and Syrian hamsters.[393] Fos-IR was increased in this nucleus in male rats following copulation,[630] and Fos-IR neurons also contained AR.[373] Female-soiled bedding elicited Fos-IR in the ventral premammillary nucleus of mice, although no increase was observed in the VMH, posterodorsal medial amygdala, or posteromedial cortical amygdala of these animals; gerbils did not show increased Fos-IR following either copulation

Nucleus Paragigantocellularis of the Medulla

Spinal mechanisms that control erection are under tonic inhibitory control. In rats,[19,638,639] mice,[20] and dogs[640] spinal transection results in an increase in the number or intensity of erections or UG reflexes or a decrease in the amount of stimulation required to elicit such responses. There is increasing evidence that a major source of that inhibition is the nucleus paragigantocellularis (nPGi) of the medulla, located in the ventrolateral medulla. Excitotoxic lesions of the nPGi facilitated copulation in male rats, indicating that resident neurons, not fibers of passage, mediated the effects.[641] Furthermore serotonergic lesions of the periaqueductal gray, which decreased 5-HT in the nPGi, also facilitated copulation.[642]

Effects of Electrolytic or Cell Body Lesions

Bilateral electrolytic lesions of the nPGi in sexually naive male rats increased the number of animals that ejaculated on their first exposure to an estrous female.[643] In the males that did ejaculate, nPGi lesions decreased the time and the numbers of mounts and intromissions preceding ejaculation and increased copulatory efficiency. In sexually experienced males, nPGi lesions increased the latency to sexual satiety and increased the number of ejaculations preceding satiety.[643] Similar lesions decreased the latency to the onset of touch-based penile reflexes and increased the number of anteroflexions.[644,645] External anal reflexes were unaffected by nPGi lesions. Instead, anal reflexes were facilitated by nucleus raphe obscurus lesions, which had no effect on erection, indicating a differential control of sexual and defecatory function.[645] Lesions of the nPGi also allowed the UG reflex to be elicited without spinal transection.[646] Indeed, nPGi lesions were as effective as spinal transection in disinhibiting the UG reflex, suggesting that the nPGi is a major source of the tonic inhibition.

Effects of Electrical Stimulation

Electrical stimulation of the nPGi of male rats has been demonstrated to activate and inhibit pudendal motoneurons.[647] It elicited field potentials in the lumbosacral cord in regions containing spinal cord neurons projecting to motoneurons of the pudendal nerve.[648] Sympathetic fibers in the pudendal nerve were specifically activated by nPGi stimulation.[649] Spinal reflex activation of pudendal neurons by stimulation of the dorsal nerve of the penis was inhibited by nPGi stimulation.[650]

Immunocytochemistry

Anatomical tracing studies demonstrated that neurons in the nPGi project directly to pudendal motoneurons in the dorsolateral and dorsomedial nuclei, sympathetic and parasympathetic preganglionic neurons in the T13-L2 and L6-S1 segments, respectively, and to the interneuronal regions of the medial gray lumbosacral cord.[644,651] A majority (78%) of nPGi neurons projecting to the lumbosacral spinal cord contain 5-HT.[196] These serotonergic neurons receive a direct projection from the PVN.[619] Animals pretreated with the serotonergic neurotoxin 5,7 dihydroxytryptamine demonstrated a decreased descending inhibition in UG reflex tests, and application of 5-HT to the spinal cord suppressed the UG reflex in spinalized rats.[458] Therefore, serotonergic input could mediate the tonic inhibition of genital reflexes.

Other Brain Areas

In a classic study, electrical stimulation of the cingulate gyrus, the medial dorsal nucleus of the thalamus, the periventricular region, and the VTA elicited erections in conscious monkeys.[652] Stimulation of the hippocampus in anesthetized rats also elicited erections.[653] The hippocampus receives excitatory oxytocinergic inputs from the PVN,[603] as well as serotonergic input from the raphe nuclei.[218,654]

Lesions of the lateral septum decreased the numbers of mounts and intromissions.[655] However, the AR antagonist hydroxyflutamide, implanted in the lateral septum of male rats, failed to prevent the T-induced restoration of copulation in castrated male rats, although similar implants into the MPOA did prevent such restoration.[391] Thus, the lateral septum may facilitate copulation, but ARs in that area appear not to contribute to its effects. Microinjection of vasopressin into the lateral septum of monogamous male prairie voles promoted pair bonding, and either a vasopressin or an oxytocin antagonist in the lateral septum blocked pair bonding elicited by either mating or microinjection of vasopressin.[656]

Lesions of the caudal zona incerta, in the subthalamic region, abolished copulation in male rats, without affecting the preference for a receptive female.[657] Thus, the zona incerta appears to be an important node in the circuit that controls copulation; however, little is known of its specific contributions.

Several cortical regions were activated during positron emission tomography (PET) scans when men were shown sexually explicit films, compared to neutral or humorous films.[80] These areas included the inferior temporal cortex, a visual association area; the right insula and right inferior frontal cortex, paralimbic areas that associate highly processed sensory information with emotional states; and the left anterior cingulate cortex, which is associated with autonomic and neuroendocrine states. Plasma T levels were correlated positively with activity in some of these areas.

Spinal Cord

The spinal cord contains the autonomic and somatic nuclei that control the hemodynamic and striated muscle effectors of erection, ejaculation, and detumescence and that provide initial processing of somatic and visceral information from the genital region. Both descending axons from the brain and local reflex loops are important for these processes (see Ref. 38 for review).

Effects of Lesions or Transections

There is convincing evidence of descending inhibition from supraspinal sources, which can be eliminated by transection of the spinal cord. Spinal cord transection releases the UG reflex from descending inhibition, allowing it to be elicited by urethral stimulation.[22] Early postnatal spinal cord transection also accelerates the developmental expression of touch-based erections in

male rats from about 38 to about 28 days of age, without altering androgen titers or their morphological correlates. Spinal cord transection also reversed the inhibition of penile reflexes resulting from dorsal penile nerve transection,[639] application of a topical anesthetic to the glans penis,[658] or excision of the penile sheath, which removes the tonic stimulation necessary for penile reflexes in neurally intact rats.[659]

Effects of Direct Application of Drugs Selective for a Neurotransmitter

The need for caution in interpreting the effects of spinal transection is emphasized by a study in which spinal anesthesia was induced in rats at the thoracic level via an intrathecal cannula.[660] After induction of spinal block, supine males exhibited the abbreviated erection latencies characteristic of spinally transected males, but unlike transected males, they had fewer and weaker erections. The reduction in erection latency may truly be due to the removal of inhibitory influences from the brain, but the decreased number and intensity of erections in spinally blocked males implies interference with excitatory influences from the brain. The increase in touch-based erections after surgical transection may therefore be secondary to neural reorganization within the cord, rather than a direct effect of removing supraspinal inhibition.

SEROTONIN

The UG reflex typically cannot be elicited unless the spinal cord is transected,[22] the nPGi is ablated,[644] or the MPOA is stimulated.[458] However, either intrathecal or ICV administration of the 5-HT neurotoxin 5,7-DHT allowed the UG reflex to be elicited without spinal transection, nPGi ablation, or MPOA stimulation.[458] This suggests that descending serotonergic axons are responsible for a major part of the central inhibition of this reflex. As noted above, a majority (78% ipsilateral) of the axons from the nPGi contain 5-HT.[644] Intrathecal administration of 5-HT abolished the UG reflex, and the 5-HT antagonist methysergide prevented the 5-HT-induced blockade of the reflex.[644] In addition, lesions of the median raphe nuclei (a major source of serotonergic neurons) significantly increased the numbers of anteroflexions (penile body erections) and cups (intense glans erections) in tests of touch-based erections.[197]

Intrathecal administration of either 5-HT or thyrotropin releasing hormone (TRH) resulted in only slight increases in mount and intromission latencies. However, co-administration of 5-HT and TRH caused a marked increase in mount and intromission latencies.[661] However, in a test of touch-based penile reflexes, intrathecal administration of TRH alone decreased the proportion of responders, decreased the numbers of reflexes, and increased their latencies.[662] TRH and 5-HT are co-localized in some bulbospinal neurons.[663] Furthermore, methiothepin, a relatively nonspecific 5-HT antagonist, partially blocked some of the inhibitory effects of TRH.[664] Therefore, either 5-HT or TRH may inhibit copulation and genital reflexes, but their effects may be enhanced by co-administration or endogenous co-release.

In contrast to the demonstrated inhibitory effects of 5-HT on genital reflexes, stimulation of one receptor subtype, the 5-HT_{2C} receptor, appears to facilitate erectile function (see the section Effects of Systematically or Intracerebroventricularly Administered Drugs) Consistent with such a facilitative effect, a systemically administered 5-HT_{2C} agonist (mCPP) facilitated erections in monkeys and rats (reviewed in Ref. 3). In anesthetized animals, the reflex response of the cavernous nerve to stimulation of the dorsal penile nerve is facilitated after the complete section of the spinal cord at the thoracic level and after systemic injection of mCPP.[214]

Intrathecal administration of 8-OH-DPAT decreased the percentage of male rats displaying touch-based erections and seminal emissions.[665] This is in dramatic contrast to the facilitative effects of intrathecally administered 8-OH-DPAT on copulation, and especially ejaculatory threshold. In the copulation tests, 8-OH-DPAT decreased ejaculation latency, intercopulatory interval, and the number of intromissions preceding ejaculation.[665] The basis for the apparent discrepancy between *in-copula* and *ex-copula* measures is not clear. However, a similar discrepancy was observed with intrathecal apomorphine (see below).

GAMMA-AMINO BUTYRIC ACID

In the lower spinal cord, GABA inhibits sexual reflexes. Intrathecal injection of the $GABA_B$ agonist baclofen into the lumbosacral (L5-S1) spinal cord decreased the number of touch-based erections and increased the latency to the first glans erection. The highest dose completely blocked penile responses.[666] However, none of the doses used prevented males from copulating to ejaculation. *In-copula* ejaculation shortly before the reflex test facilitated the onset of touch-based erections in saline controls and also blocked the inhibitory effects of the lower two doses of baclofen, but not the highest dose. Therefore, as in the experiment using 8-OH-DPAT,[665] different neural mechanisms appear to control copulation and *ex-copula* reflexes. In contrast to the inhibitory effects of intrathecal baclofen on *ex-copula* reflexes, the $GABA_A$ agonist THIP produced only slight inhibitory effects at the highest dose. Therefore, stimulation of spinal $GABA_B$, but not $GABA_A$, receptors inhibits *ex-copula* reflexes.

DOPAMINE

Intrathecal infusion of lisuride, a nonspecific monoamine agonist, into the region of the rostral lumbar spinal cord of male rats produced the same effects on copulation as seen following systemic injection, namely,

decreased time and number of intromissions preceding ejaculation.[667] Therefore, stimulation of some type(s) of monoamine receptors appears to facilitate ejaculation, perhaps by increasing sensitivity to peripheral stimulation. Furthermore, intrathecal administration of the catecholamine neurotoxin 6-OHDA increased postejaculatory intervals of male rats and rendered the animals more sensitive to androgen deprivation.[668]

Intrathecal administration of the DA agonist apomorphine also decreased the number of intromissions preceding ejaculation, suggesting that stimulation of spinal cord DA receptors facilitated ejaculation.[668a] However, intrathecal apomorphine also increased the interintromission interval, the ejaculation latency, and the postejaculatory interval. In touch-based reflex tests, the highest dose of intrathecal apomorphine decreased the numbers of touch-based erections and anteroflexions but did not affect seminal emissions.[668a] These data stand in contrast to the effects of systemic injection of apomorphine, which facilitated erections at low doses, inhibited erections at high doses, and facilitated seminal emissions at both low and high doses.[148] However, in anesthetized rats, intracavernous pressure was increased by apomorphine administered intrathecally.[669] The explanation for the different results from these studies is unclear.

NOREPINEPHRINE

Intrathecal administration of NE increased the frequency of pelvic thrusting during intromission, whereas intrathecal administration of both the α_2 agonist clonidine and the β agonist isoproterenol decreased the frequency of thrusting.[670] It is likely that clonidine's inhibitory effect resulted from stimulation of α_2 autoreceptors, thereby decreasing NE release. These results are consistent with the findings that α_1 receptors probably mediate NE's facilitative effects on copulation.

OXYTOCIN

Administration of oxytocin at the lumbosacral, but not the thoracolumbar, level increased intracavernous pressure in dose-dependent fashion.[669] The proerectile effects of intrathecal oxytocin were blocked by an oxytocin antagonist, pelvic nerve section, and a nicotinic antagonist, which blocked transmission between the pre- and postganglionic parasympathetic nerves. An oxytocin agonist also produced proerectile effects. Intrathecal administration of a NO inhibitor blocked the pro-erectile effects of intrathecally delivered oxytocin, indicating that the effects of oxytocin in the spinal cord are mediated through NO interneurons. Vasopressin, a nonapeptide that differs from oxytocin by only one amino acid, was ineffective, as were lumbosacral saline and systemic oxytocin. Blockade of striated muscle activation failed to inhibit oxytocin's facilitative effects, suggesting that oxytocin's effects were mediated by the parasympathetic nervous system, and not by striated penile muscles. These data provide compelling evidence for the proerectile role of oxytocin-containing fibers descending from the PVN to the lumbosacral cord.

OPIATES

Intrathecally administered naloxone decreased the number of intromissions preceding ejaculation, whereas morphine administered in a similar manner increased the number of intromissions.[671] These treatments had no other effects on copulation. The authors suggested that naloxone and morphine influenced the strength of the sensory signal from each intromission.

NITRIC OXIDE

As noted in the section on MPOA stimulation, intrathecal administration of an NO donor, a cGMP analog, or the phosphodiesterase-V inhibitor sildenafil enhanced the increase in intracavernous pressure elicited by electrical stimulation of the MPOA; the NOS inhibitor L-NAME reduced the response.[457] None of these treatments affected blood pressure or intracavernous responses to cavernous nerve stimulation. The authors concluded that central NO/cGMP can affect erectile capacity.

Chemical Changes Detected by Analysis of Cerebrospinal Fluid

The concentration of GABA in cerebrospinal fluid of male rats increased by more than 1000% following ejaculation; concentrations of aspartate and glutamate increased by about 200%.[672] Therefore, the concentration of a major inhibitory neurotransmitter increased markedly during the absolute refractory interval.

Presence of Steroid Receptors

AR-IR was found in three areas of the male rat lumbosacral spinal cord: the spinal nucleus of the bulbocavernosus (SNB), dorsolateral nucleus (DLN), and retrodorsolateral nucleus (RDLN).[673] Virtually no AR-IR was seen in long-term castrated males or males with the Tfm mutation, which renders the AR inactive. Furthermore, unilateral capsules filled with T and placed next to the bulbospongiosus muscle, increased the dendritic arborization of SNB neurons of castrated male rats. In contrast, testosterone administered to the spinal cord of castrated rats was ineffective.[674] Thus, despite the presence of AR in the pudendal motoneurons, the trophic effect of androgens is mediated by actions on the target muscle.

Gréco et al.[675] also found dense labeling for AR in the L5-S1 segments of the spinal cord, especially in the dorsal part of Lamina X. Furthermore, mating-induced Fos-IR was predominantly located in AR-IR neurons.

Therefore, the lumbosacral neurons that are activated by mating are androgen sensitive.

Immunocytochemistry

The 5-HT_{2C} receptor at the spinal level appears to facilitate erectile function. Consistent with such a facilitative effect, 5-HT_{2C} receptor immunoreactivity was found on motoneurons of the sacral parasympathetic nucleus, the dorsal gray commissure, and the motoneurons of the ventral horn.[676] Furthermore, 5-HT_{2C} receptor immunoreactivity was exhibited by all neurons retrogradely labeled from the corpus cavernosum to the sacral parasympathetic nucleus and the dorsal gray commissure of L5-L6, and in ventral horn motoneurons retrogradely labeled from the ischiocavernosus and bulbospongiosus muscles. Therefore, the neurons that are known to promote erection possess 5-HT_{2C} receptors.

Immunocytochemical studies revealed that dopaminergic fibers and terminals exist in virtually all laminae throughout the spinal cord.[677] D_2 receptors identified by immunohistochemical and in situ hybridization techniques were noted in the parasympathetic area of the lumbosacral spinal cord and found to be particularly abundant in the dorsomedial and dorsolateral motoneurons innervating the bulbospongiosus and ischiocavernosus muscles.[678]

Neurons and fibers immunoreactive for α_{2a} and α_{2c} adrenoceptor subtypes were identified in the intermediolateral cell column, the dorsal gray commissure, and the ventral horn of the T12-L2 and L5-S1 spinal cord. The dorsal horn displayed only α_{2a}-IR fibers. Pseudorabies virus-infected neurons in the autonomic nuclei were immunoreactive for both α_{2a} and α_{2c} adrenoceptor subtypes and closely apposed by α_{2a} and α_{2c} immunoreactive fibers.[679] These results suggest an intraspinal modulation of the noradrenergic and adrenergic control of the autonomic outflow to the penis by pre- and postsynaptic α_2 adrenoceptors.

Oxytocinergic axons from the PVN densely innervate clusters of sympathetic preganglionic neurons in the intermediolateral cell column of the thoracolumbar spinal cord.[680,681] There is also immunoreactivity for oxytocin in the sacral parasympathetic nucleus of the rat spinal cord, the dorsal horn, and the dorsal gray commissure. Oxytocin fibers make synaptic contacts on preganglionic neurons in the sacral parasympathetic nucleus.[681] Oxytocin receptor binding was found in the superficial dorsal horn, sacral parasympathetic nucleus, and dorsal gray commissure of the male rat lumbosacral spinal cord.[682]

A coital reflex observed in rats that were spinally transected at the T6 level includes rhythmic muscular activation and expulsion of semen from the urethra.[683,684] The reflex could be elicited multiple times by penile stimulation, but the muscle after-discharges and the penile movements decreased after the first reflex, and the reflex was exhausted after six or seven occurrences. The authors noted that copulating male rats also become sexually exhausted after about seven ejaculations. The spinal generator of ejaculation includes neurons in segments L3 and L4 of the lumbar spinal cord that contain both galanin and CCK[685] and project to the SPFp of the thalamus.[43] These lumbar spinothalamic (LSt) neurons are thought to relay somatosensory information concerning ejaculation from the genitals to the brain. Galanin and CCK immunoreactive neurons showed increased Fos expression only in males that had ejaculated one or two times and not in animals that only mounted or intromitted (except for a few Fos-IR neurons in one male that only intromitted).[43] Furthermore, lesions of galanin-containing neurons in L3 and L4 severely impaired ejaculatory ability of male rats.[43] Therefore, these neurons not only relay ejaculation-specific sensory information to the brain, but also contribute to the execution of the behavior. Lesions of these neurons resulted in a loss of the ejaculatory reflex in spinally transected rats.[686] Thus a spinal generator of ejaculation, which includes the LSt neurons, is normally under inhibitory control from higher neural areas.

CIRCUITRY AND ANATOMICAL INTERCONNECTIONS

Thus far, we have considered separately the functions and connections of different brain nuclei in the control of male sexual behavior. However, it is probably more accurate to view each region of the brain as belonging to one or more functional circuits that converge on the MPOA. In terms of afferent input, MPOA receives chemosensory cues from the vomeronasal organ and olfactory mucosa, somatosensory stimuli from the genitals, as well as direct and indirect hormonal input via steroid receptor-containing neurons. There are also excitatory and disinhibitory pathways from the MPOA to the midbrain and brainstem, from which neurons descend to the spinal cord nuclei to regulate genital reflexes and copulatory motor patterns.

A great deal of research is directed at understanding the signals and pathways through which individual sensory and endocrine stimuli influence mating behavior. However, sensory and humoral cues are seldom presented in isolation. Instead, an organism in a natural setting is confronted with multiple signals from its internal and external environment that together are interwoven to yield an experience richer than the sum of the individual modalities.[687]

To understand complex motivated behaviors, we must determine how different cues are weighed,

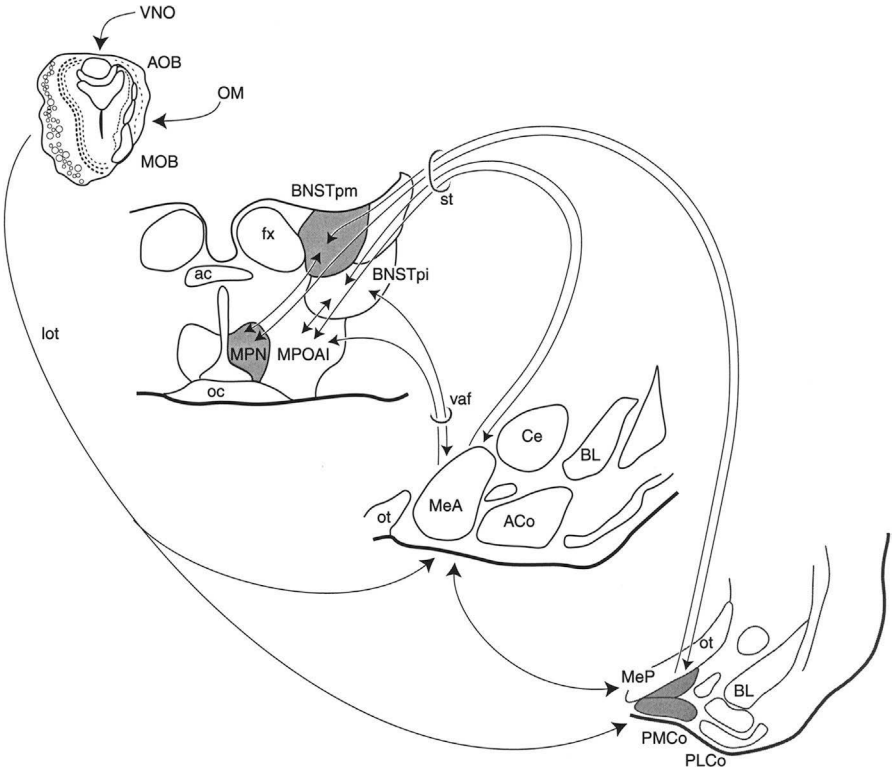

FIGURE 49.23 Schematic diagram of principal limbic nuclei and connections in hamster brain that transmit chemosensory and hormonal cues to control male sexual behavior. Shading indicates areas with abundant steroid receptor-containing neurons. ac, anterior commissure; ACo, anterior cortical amygdaloid nucleus; AOB, accessory olfactory bulb; BL, basolateral amygdaloid nucleus; BNST, bed nucleus of the stria terminalis; BNSTpi, posterointermediate subdivision of BNST; BNSTpm, posteromedial subdivision of BNST; Ce, central amygdaloid nucleus; fx, fornix; lot, lateral olfactory tract; Me, medial amygdaloid nucleus; MeA, anterior subdivision; MeP, posterior subdivision; MOB, main olfactory bulb; MPN, medial preoptic nucleus; MPOAl, lateral subdivision of the medial preoptic area; oc, optic chiasm; OM, olfactory mucosa; ot, optic tract; PLCo, posterolateral cortical amygdaloid nucleus; PMCo, posteromedial cortical amygdaloid nucleus; st, stria terminalis; vaf, ventral amygdalofugal pathway; VNO, vomeronasal organ. *Source: Reprinted from Ref. 688, with permission.*

prioritized, and integrated. The specific signals to guide sexual activity are tailored to the biology of the species. Although rodents rely principally on chemosensory stimuli to initiate behavior, other species use visual, auditory, or somatosensory stimuli, or a combination of sensory cues. Nonetheless, multimodal integration of sensory and humoral signals is ubiquitous, and further studies may uncover common mechanisms that guide the behavior of diverse species.

The Chemosensory Circuits

Lesions along the chemosensory circuit that eliminate mating have established the importance of the Me, BST, and MPOA in male sexual behavior (Figure 49.23). Our understanding of this circuit is enhanced through the use of Fos to identify activated neurons, including steroid-responsive neurons. However, the activity of the chemosensory circuit is also modified by hormonal stimuli. Me, BST, and MPOA each contain large numbers of steroid receptor-containing neurons, and intracerebral implants of T in either Me or MPOA enhance sexual activity above castrate levels.[393,467] These effects are mediated by aromatization to E, for implants of DHT in either area are without effect.[386,689] Steroid implants not only facilitate male copulatory behavior, they also enhance the response to odor cues. Specifically, male mice with intracerebral implants in the MPOA will make ultrasonic vocalizations to female urine.[561] Likewise, intracerebral T implants in Me or BST/MPOA of male hamsters stimulate anogenital investigation.[393] Together, these studies suggest that steroid action along the chemosensory pathway facilitates sexual behavior and responsiveness to sexually relevant chemosensory stimuli.

The challenge is to understand how steroid hormones interact with chemosensory cues to regulate male sexual behavior. Cottingham and Pfaff[394] have proposed that steroids act as a gating mechanism to permit or enhance transmission of chemosensory cues through steroid-sensitive brain nuclei. At the cellular level, castration may reduce synaptic efficiency in the transmission of odor cues by inhibiting release of neurotransmitters and/or suppressing postsynaptic receptors. Gonadal steroids also promote growth and branching of neurons. Although these effects are most dramatic during development, steroids also stimulate neuronal growth in the adult, as detailed by Garcia-Segura et al.[690] In the male Syrian hamster, castration reduced the mean somal area, highest dendritic branch, and percentage of neurons in Me with tertiary branches.[691] There is also potential for steroid effects on synaptic connections. Changes in connectivity can take place rapidly, in the absence of substantial alterations in gross neuronal structure. This has been best studied in females, where synapses in the arcuate nucleus of the hypothalamus are remodeled over the course of the four-day estrous cycle.[692] In the male, loss of synaptic connections after castration could have a major impact on transmission of chemosensory cues

through Me, BST, and MPOA. It is likely that both structural and neurochemical mechanisms contribute to the decline in sexual behavior after castration.

Somatosensory Input from the Genitals

The dorsal nerve of the penis (DNP), a branch of the pudendal nerve, carries sensory input from the penile skin, prepuce, and glans penis.[347] It is the major source of afferent input from the penis, although the cavernous nerve also contributes input from deeper structures.[347] The afferents terminate primarily in the medial portions of the dorsal horn and in the medial central gray matter (dorsal gray commissure) of the lumbosacral spinal cord. Sensory information is relayed to supraspinal sites via both spinothalamic and spinoreticular pathways. The spinothalamic pathways primarily convey the fastest fibers related to the encapsulated nerve endings of the penis. They travel in the dorsal columns and consist primarily of fast myelinated fibers. These fibers terminate in the posterolateral nucleus of the thalamus, and input is subsequently relayed to the medial thalamus. Spinoreticular fibers tend to be slower than the spinothalamic fibers. They travel in the contralateral (and to a lesser extent ipsilateral) lateral spinal columns and terminate in the brainstem reticular formation.

Electrical stimulation of the DNP elicited activity in the nPGi, the PVN, the MPOA, and the cortex.[693,694] The CTF of male rats and SPFp of rats, gerbils, and hamsters show increased Fos-IR following copulation and also project to the MPOA (reviewed in Ref. 3). Finally, a combined anterograde and retrograde tracer study revealed that axons from the L3 and L4 segments of the lumbar spinal cord terminated in close proximity to neurons that projected to the MPOA or BSTpm.[695]

Studies in humans have examined cortical evoked potentials following electrical stimulation of the dorsal nerve of the penis/clitoris (a division of the pudendal nerve). Evoked potentials were recorded bilaterally from cortical areas, with the highest amplitude in the midline (Cz-2) over the sensory cortex.[696] This distribution is consistent with pudendal representation deep in the midline interhemispheric fissure in humans[696a] and cats.[697] The amplitudes of cortical evoked responses were larger in men than in women[693] although slightly shorter in latency in women.[698] The smaller size in women may be related to fewer fibers innervating the clitoris relative to the penis or to the greater accessibility of stimulation of the male dorsal nerve.

An Ejaculation-Related Circuit

As noted above, a spinal ejaculation generator that includes LSt neurons and projects to the SPFp is under supraspinal inhibitory control. Ejaculation, but not copulation without ejaculation, elicited small clusters of Fos-IR in the MePD, the BSTpm, posterodorsal preoptic nucleus (PdPN), and the SPFp of rats, hamsters, and gerbils (reviewed in Ref. 3). Using anterograde and retrograde tracing techniques, it has been demonstrated that neurons in each of these areas expressing ejaculation-induced Fos are reciprocally connected with the MPOA (Figure 49.24).[538,624] In addition, combined unilateral lesions of MePD and SPFp in rats[337] and lesions of the SPFp in gerbils[358] resulted in decreased mating-induced Fos expression in the MPOA, demonstrating that these inputs contribute to the neural activation of the MPOA.

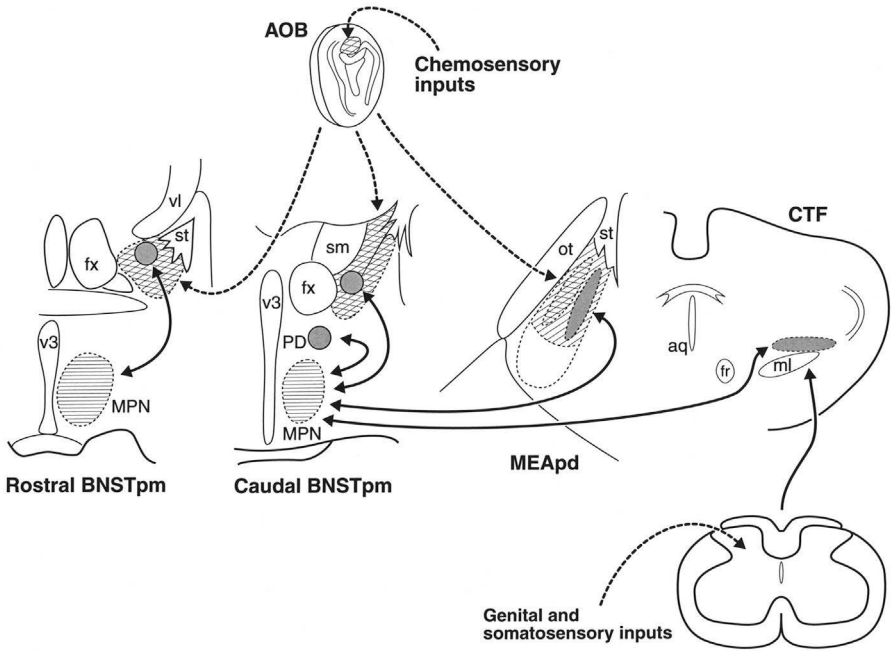

FIGURE 49.24 Schematic overview of neural activation in circuits underlying male sexual behavior. Areas where Fos is induced following chemosensory cues or chemosensory investigation are illustrated by diagonal stripes from upper left to lower right. Areas where Fos is induced primarily following ejaculation are illustrated in dark shading. Areas where Fos is induced by all consummatory elements of behavior are illustrated by diagonal stripes from lower left to upper right. AOB, accessory olfactory bulbs; MPN, medial preoptic nucleus; PD, posterodorsal preoptic nucleus; BNSTpm, posteromedial bed nucleus of the stria terminalis; MEApd, posterodorsal medial amygdala; CTF, central tegmental field; LSSC, lumbosacral spinal cord; v3, third ventricle; fx, fornix; vl, lateral ventricle; st, stria terminalis; sm, stria medularis; ot, optic tract; aq, aqueduct; Fr, fasciculus retroflexus; ml, medial lemniscus. *Source: Figure reproduced from Ref. 41, with permission.*

However, the majority of ejaculation-activated cells in the MePD, PdPN, or mSDA of gerbils did not project to other members of the ejaculation-related circuit; the authors suggested that the majority of the Fos-IR neurons either were interneurons or projected to other brain areas.[538] Indeed, a later article reported that ejaculation-activated neurons in the PdPN and MePD projected to the anteroventral periventricular nucleus (AVPV), the dorsal portion of the principal part of the BST, and the retrorubral field.[699]

It is not clear whether the activated neurons primarily received sensory input or activated motor patterns leading to ejaculation. The similar pattern of Fos-IR in female rats following ejaculation by the male[700,701] suggests that these neurons are activated by sensory input rather than programming motor output. However, lesions of the PdPN or MePD decreased mounting and delayed ejaculation.[358] Thus, these data suggest a contribution of these structures to the motor control of copulation, but not a major specific role in triggering ejaculation. Lesions of the posterior thalamus that included SPFp and zona incerta completely disrupted ejaculatory behavior in male rats,[702] supporting a role for the posterior thalamus in control of ejaculation. In contrast, small lesions restricted to the SPFp in gerbils had no effect on copulation,[358] suggesting that the SPFp may relay copulation-related information, rather than triggering ejaculation. The SPFp receives ascending sensory inputs, either directly or indirectly, from the lumbosacral and cervical regions of the spinal cord, and in turn projects to the BSTpm and MePD of rats.[703] In gerbils the SPFp projects to the mSDA, PdPN, MePD, but does not receive reciprocal input from those structures.[699]

Yahr and her colleagues have provided information about the neurotransmitters used by ejaculation-activated neurons in gerbils. One-third to one-half of Fos-IR neurons in the lateral PdPN and MePD expressed NOS; in addition, half of the Fos-IR neurons in the MePD, lateral PdPN, and medial sexually dimorphic nucleus of the MPOA (mSDA) were GABAergic, and a quarter of those in the MePD and mSDA were glutamatergic; the PdPN appeared to have no glutamatergic cells.[380] The authors suggested that these neurons may influence the timing of ejaculation, the length of the postejaculatory interval, or release of LH or prolactin at the time of ejaculation.

Male hamsters show Fos activation in the BSTpm and MePD only after multiple ejaculations, or when they are reaching sexual satiety after only a few ejaculations, due to mating on previous consecutive days.[363] Moreover, small lesions directed at the lateral MePD increased the number of ejaculations necessary for satiety.[704] Hence, the MePD may contribute to ejaculatory behavior and promote sexual satiety in addition to other aspects of male sexual behavior.

Lesions of the lateral hypothalamus severely affect the display of ejaculations, but do not affect the ability to mount and intromit.[557] Thus, this area may play an excitatory role in the regulation of ejaculation. Interestingly, 5-HT is released in the anterior LHA at the time of ejaculation,[533] and microinjection of a selective 5-HT reuptake inhibitor into the LHA increased the latencies to mount, intromit, and ejaculate.[533] Hence, 5-HT in the LHA may influence sexual motivation as well as ejaculatory behavior.

A study by Holstege and coworkers[419] used positron emission tomography to study increases in regional cerebral blood flow during ejaculation in men. Increased blood flow was observed following ejaculation compared to sexual stimulation in the mesodiencephalic junction, which includes the location of the SPFp. In addition, increased blood flow was observed in cerebellum, lateral putamen, claustrum, and several cortical areas. Increased blood flow was not detected in the MPOA or BNST, which contrasts with studies in rodents but is in agreement with a study in male macaques that failed to detect Fos activation in the MPOA following ejaculation.[409]

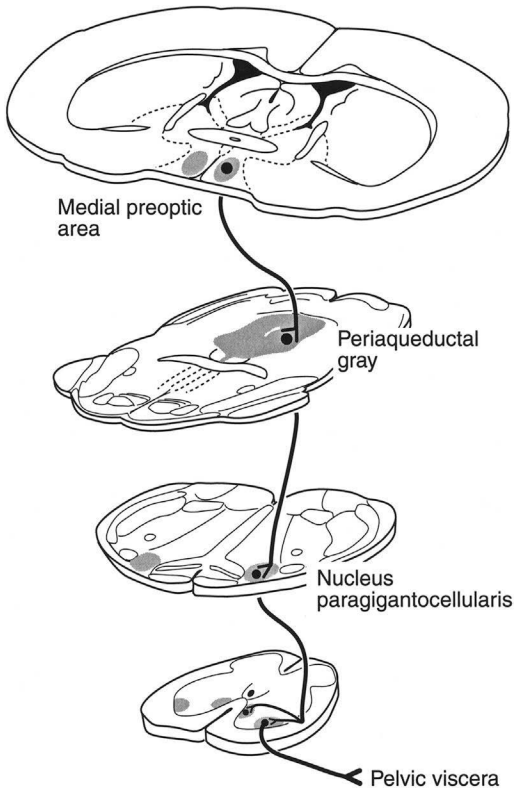

FIGURE 49.25 Summary schematic showing the MPOA-PAG-nPGi-spinal cord circuit. MPOA projections to the periaqueductal gray (PAG) terminate preferentially among PAG neurons projecting to the nucleus paragigantocellularis (nPGi). Descending projections from the nPGi terminate within the dorsomedial and dorsolateral motor pools of the ventral horn of the lumbosacral spinal cord. Motoneurons from these pools innervate the bulbocavernosus and ishiocavernosus muscles, which are essential for penile erection and ejaculation. *Source: Figure is from Ref. 633, with permission.*

Efferents from the MPOA

The primary efferents from the MPOA travel through the medial forebrain bundle to the midbrain.[705] However, we should emphasize that most of the afferent and efferent connections of the MPOA with other brain regions are reciprocal.[424] Therefore, "downstream" structures can both modulate MPOA processing and receive its output. Evidence for the importance of axons leaving the MPOA laterally to join the medial forebrain bundle include reports that knife cuts lateral to the MPOA disrupted copulation similarly to lesions of the MPOA itself, whereas knife cuts either rostral or caudal to the MPOA were ineffective.[706] Similarly, lesions along the extent of the medial forebrain bundle caudal to the MPOA permanently eliminated copulation, whereas lesions rostral to the MPOA did not affect copulation (reviewed in Ref. 3). Furthermore, contralateral lesions of the MPOA and medial forebrain bundle were as effective as bilateral lesions of either structure.[707]

The critical axons in the medial forebrain bundle provide reciprocal connections between the MPOA and the ventral pons and medulla (Figure 49.25).[708] Dense projections from the MPOA reach the nPGi and midline raphe nuclei. Lesions of the nPGi facilitated copulation,[643] touch-based erections and anteroflexions,[644] and the UG reflex.[646] 5-HT from the raphe nuclei is also generally inhibitory to male sexual behavior (reviewed in Ref. 576). Therefore, one means by which the MPOA can facilitate sexual behavior is by inhibiting these two areas. However, these areas may also send reciprocal input to the MPOA to inhibit sexual behavior. The MPOA also provides dense innervation to the PAG, which in turn projects to the nPGi. Thus, the MPOA can influence the nPGi through both direct and indirect connections. The PAG has numerous AR- and/or ER-containing neurons.[636] Furthermore, both the MPOA efferents to the PAG and the PAG efferents to the nPGi contain AR and/or ERα, and axons from the MPOA end in close juxaposition to PAG-to-nPGi efferents.[633] Therefore, gonadal hormones could affect the ability of the MPOA to disinhibit sexual behavior. However, it is unlikely that mere disinhibition can account for the elicitation of reflexes that has resulted from electrical or chemical stimulation of the MPOA (reviewed in Ref. 3). It seems likely that there is also a pathway that provides excitation to the spinal neurons that control the reflexes. Peripheral pathways that mediate that excitatory influence appear to include activation of both parasympathetic outflow, via the pelvic and cavernous nerves, and sympathetic efferents, via the paravertebral sympathetic chain and possibly the hypogastric nerve.[39] Although the parasympathetic system provides the main pro-erectile force, the sympathetic system may also contribute, perhaps by producing vasoconstriction in nongenital areas, thereby diverting blood to the penis.[39] (See the section Neural Innervation of the Penis earlier in the chapter.)

Sexual Behavior in the Context of Mammalian Social Behavior

The same brain areas that control male sexual behavior also regulate other social behaviors, including female sexual behavior, maternal behavior, aggression, and territorial marking. Newman[405] has suggested that these structures form a richly and reciprocally interconnected circuit subserving mammalian social behaviors. Most of the areas, except the midbrain, are copiously populated with steroid receptors, and all contribute to more than one behavior. Perinatal, adolescent, and adult hormones mold sexually dimorphic responsiveness to social stimuli. Substructures of these brain areas may be specialized for specific behavioral components. However, the circuit that is activated specifically by ejaculation in male rats, hamsters, and gerbils, also responds in female rats to ejaculation by a male. Therefore, this circuit may either "count" stimuli or regulate timing in both males and females to promote a successful pregnancy. It is unclear whether the same neurons contribute to different behaviors or whether behavior-specific neurons within each structure mingle with neurons specific for other behaviors. Organizational and activational effects of hormones may confer specificity in response to stimuli and thereby promote diversity in the network.

CONCLUSION

Our understanding of the neural mechanisms of male sexual behavior is based primarily on our understanding of the rat and other animal models. Nevertheless, as is evidenced in this chapter, there is considerable homology among species with regard to sex-relevant mechanisms, which permits us to extrapolate from the animal data while informing our understanding of the human condition.

The neural mechanisms of male sexual behavior are organized developmentally by hormones, primarily estrogens in rodents and primarily androgens in primates, including humans, and in guinea pigs. Those neural circuits are then activated by the same hormones, beginning in puberty. Sensory circuits relay information about sexually relevant stimuli to several brain areas that integrate those stimuli and program appropriate motor output. However, those integrative areas also send output back to the sensory areas to amplify the relevant sensory signals. Hormones have primarily slow, genomically mediated effects, including production of enzymes, receptors, and structural proteins, although they may also have rapid effects via membrane-associated receptors.

Chemosensory input arrives via the main and accessory olfactory systems and is processed by the medial and cortical areas of the amygdala and the BST; activation of hormone receptors is critical for the responsiveness of those areas. The amygdala and BST also receive sensory input from the genitals via the central tegmental field of the midbrain. The amygdala and BST also receive visual and auditory information.

The MPOA indirectly receives input from all sensory systems and sends reciprocal output back to those areas. Thus, hormone-concentrating neurons can bias the sensory input to favor sexually relevant information. Although the MPOA has primarily parasympathetic connections, which promote erections, it can also indirectly activate some sympathetic neurons, which can elicit seminal emission and ejaculation and also contribute to erections. DA is released in the MPOA as soon as the male detects the presence of an estrous female and is critical for mating to occur. Glutamate from the amygdala and BST activate production of NO, which is necessary for female-stimulated DA release and for copulation. A large spike of glutamate is released at the time of ejaculation. E2 is necessary to maintain NO synthase, which in turn maintains basal DA levels, but androgen is necessary for the DA increase in response to the female and for optimal copulation.

The PVN, which is caudal to the MPOA, can also elicit erections and ejaculation. It appears to be more important for NCE, elicited by the sight or smell of an estrous female, than for copulation itself, though it also contributes to copulation. DA, oxytocin, excitatory amino acids, and hexarelin peptides are excitatory to NO-producing neurons in the PVN, which release oxytocin in the hippocampus, pons, medulla, and spinal cord to stimulate erection and ejaculation. GABA and opioid peptides inhibit those neurons.

Other hypothalamic areas that contribute to mating include the perifornical LH, the site of orx/hcrt neurons, which project to both the MPOA and the mesocorticolimbic DA tract and activate those areas. 5-HT is released in the perifornical LH at the time of ejaculation and inhibits the orx/hcrt neurons, thereby inhibiting copulation. Activation of those 5-HT receptors would likely contribute to the sexual dysfunction that often results from SSRI antidepressants, although there are also inhibitory 5-HT receptors in the spinal cord, which normally receive input from the nPGi.

The mesocorticolimbic DA tract originates in the midbrain tegmentum and projects to the Acb and the prefrontal cortex. It is sometimes referred to as the "engine" that drives numerous motivated behaviors, including mating. DA is released in the Acb as soon as a male rat detects the presence of an estrous female, suggesting that it contributes to sexual motivation, not just motor activation. On the other hand, the nigrostriatal DA tract is activated only during actual copulation, suggesting that it is less important for sexual motivation. Oxytocin in the VTA appears to stimulate the DA-ergic neurons, and CCK released in the Acb potentiates the facilitative effects of DA. However, 5-HT agonists microinjected into the Acb inhibit ejaculation.

The PAG contains steroid receptors and receives output from the MPOA; in turn, it projects to the nPGi, which sends inhibitory 5-HT-containing axons to the spinal cord. Stimulation of the MPOA can override the normal inhibition of the UG reflex, apparently by inhibiting the nPGi.

The spinal cord provides initial processing of somatic and visceral information from the genitals and the autonomic and somatic nuclei that control the hemodynamic processes and striated muscles that produce erection, ejaculation, and detumescence. Both descending axons from the brain and local reflex loops are important for these processes.

The brain and spinal areas are heavily interconnected and form circuits that specialize in chemosensory, genitosensory, visual, and auditory inputs that elicit mating and that elicit approach to a female and trigger erection and ejaculation.

Answers to previous questions inevitably raise new questions to guide future research. These questions fall into several broad categories: anatomical interconnections, immediate and long-term neurotransmitter effects and their interactions, electrophysiological responses, roles of intracellular messengers and gene expression, similarities and differences within and between species in the control of male sexual behavior, and in the control of other social behaviors.

Questions concerning anatomical interconnections include the following: By which pathways do the main and vomeronasal olfactory systems promote sexual arousal? How do the pathways that control noncontact, touch-based, and *in-copula* erections differ and where do they converge? Similarly, what are the similarities and differences between pathways that elicit the UG reflex and *in-copula* ejaculation? Which neural efferents from the MPOA carry the disinhibitory and the excitatory influences on genital reflexes? What pathways mediate the parasympathetic and sympathetic proerectile effects of MPOA stimulation?

Issues related to neurotransmitters include: Which neurotransmitters, and which receptors, mediate the major effects in each of the sensory, integrative, and motor control sites? Which neuropeptides are co-released with classic transmitters, and under which conditions? Do changes in neurotransmitter release reflect primarily changes in cell firing or influences on presynaptic terminals? Which (combinations of) neurotransmitters elicit long-term changes in sensitivity, as opposed to immediate responses to stimuli?

Questions related to intracellular and genetic influences include the following: Which ion channels and/or second messengers mediate the immediate and long-term effects of neurotransmitters in brain areas that respond to sexually relevant stimuli? What changes in gene transcription are elicited by steroid hormones binding to classic steroid receptors? What are the effects on sexual behavior of rapid membrane responses to steroid hormones? How does sexual experience affect gene transcription? How does activation of immediate early genes facilitate subsequent sexual behavior? Are new neurons recruited by sexual experience, or are the facilitative effects of experience mediated by strengthening of previous connections? What do patterns of electrophysiological responses contribute to the neural control of copulation?

Questions concerning broader issues include: How do general activational systems, such as the mesocorticolimbic system, mesh with systems that promote specific behaviors? What *does* the MPOA do? What differences in neuroanatomical and physiological processes result in species differences in behavior? How are behavioral differences between species related to ecological niches? How are the neural elements of male sexual behavior similar to and different from those that control other social behaviors?

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CHAPTER

50

Female Sexual Behavior

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INTRODUCTION

Interest in female sexual behavior and reproduction is likely as old as our species. Indeed, one of the oldest known medical texts, the Kahun Papyrus, found near the pyramid in El-Lahoun, Egypt, in 1889 and dated to at least 1800 BCE, is a gynecological text that outlines causes and treatments for women's reproductive health problems, including problems of fertility, contraception, and pregnancy.[1] Ancient erotic texts, such as Vatsayana's *Kama Sutra* (compiled from Hindu writings in the second century BCE), Ovid's *Ars Amatoria* (c.1 BCE), and the Taoist *Art of the Bedchamber* books (compiled between 206 BCE and 24 CE), devoted large portions to understanding how to court women properly; how to stimulate arousal, desire, and pleasure; and how women's receptivity to such courting and stimulation shifted across the menstrual cycle.[2] The striking similarity in descriptions, despite vast differences in culture, suggests some degree of commonality in the way women's sexual physiology and psychology are constructed and altered, respectively, by ovarian hormones.

The *Hippocratic Corpus*, dating from the fifth century BCE, contains seven early volumes written by Hippocrates himself that were concerned with women's fertility and diseases specific to women.[1] The first of these contains an oddly modern account of midcycle rises in women's sexual lust and receptivity, driven of course by the lunear impact on women's "psychic pneuma" (the four endogenous fluids—blood, yellow bile, black bile, and phlegm—that were thought to mediate the four basic emotions of passion, anger, sadness, and calm). Hippocrates also notes that "hysteria" should be treated by gentle stimulation of the clitoris and/or vagina to orgasm (or sexual intercourse, if the woman is married or has a knowledgeable sex partner). It was believed that orgasm would center the uterus back into its normal position. The second contains a then-comprehensive list of contraception methods, including the use of small scrubbed stones as intrauterine devices. This practice was based on the experiences of Egyptian and Bedouin camel drivers, who routinely placed stones in the uteri of camels to prevent pregnancy. It is likely that animal sexual behavior and reproduction have been studied since humans started domesticating animals, but largely from a veterinary and/or animal husbandry perspective, and only in those species used as pack animals (camels, sheep, donkeys), for agricultural work (e.g., oxen, horses), or for food (e.g., sheep, cattle). In his work *Historia Animalium*, Aristotle (384–322 BCE) described the routine spaying (ovariectomy, OVX) of adult sows and camels as common agricultural practice, done specifically to eliminate sexual behavior and control reproduction.[3]

Rudimentary psychological understanding of sexual arousal, desire, pleasure, and inhibition in women can be found in art and prose, from ancient religious stories (e.g., "Song of Songs"), plays (e.g., Shakespeare's "The Taming of the Shrew"), to myriad love poems written throughout the ages, mostly by men about women, but also written by women (e.g., the "nine earthly muses"

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of Ancient Greece, notably Sappho and Praxilla who wrote erotic, comic, and tragic poetry about the sexual desires of women and men). Most ancient polytheistic cultures had women depicted as goddesses of sex and fertility. The Aztecs had eight deities—seven of which were women—who controlled the intricacies of sex and reproduction. However, goddesses of love and carnality were associated with freedom, war, or mental illness. An example of the latter was the Moroccan lust goddess, Qandisa, who seduced men and then drove them insane.

Erotic art has existed for a very long time, starting with cave drawings of copulation (often between animals, but also heterosexual and homosexual copulation between adult humans). Fertility statues also exist, dating back well over 10,000 years, and almost always depicting a full-bodied, reproductively capable woman (e.g., the Venus of Willendorf). Erotic art from India, Japan, Greece, and Rome during ancient times depicts a variety of imaginable sex acts between two or more humans, humans and animals, and even humans and inanimate objects. Women were depicted masturbating and initiating sex. This did not change in Europe during the Middle Ages, Reformation, or Enlightenment periods, as Néret's[4] *Erotica* series depicts. Women are drawn by many artists with smiles and in full control of sexual interaction, again often initiating sex and in positions (e.g., female supine) that would maximize clitoral and inner vaginal stimulation. Women's orgasm was thought to be positively related to fertility; thus, hints of masturbation were found in artistic works throughout these periods. For example, Titian's "Venus of Urbino" of 1538 depicts her reclining sensually with her hand draped over her mons, her fingers easily in position to stimulate her clitoris. Ēdouard Manet's "Olympia" of 1863 has a similar depiction, this time with her hand outstretched over her pubis, but her forefinger hidden seductively between her legs. There is also a large portrayal of women in fetish circumstances during the eighteenth century, often controlling the man in the action. This is in stark contrast to the portrayal of women in a large part of nineteenth and twentieth century pre-Internet erotica as being either passive exposers of their genitalia or passive and emotionless recipients of male penetration.[5]

As science and rationalism took hold in Europe and the Americas, so did attempts at understanding behavior based on deductions about underlying physical causes. For female sexual behavior, this began with the nineteenth century phrenologists who attempted to understand the nature of female sex drive as a function of the size of bumps on the skull that they believed reflected the size of the underlying brain structure. Among these was the so-called amativeness center at the back base of the skull, just over the cerebellum, that allegedly controlled sexual and parental instincts in women.[6]

Early ethologists began to categorize reproductive behavior in the late 1800s. Notable among these descriptions was that of Darwin,[7] who discussed sexual selection in terms of flexible *female* choice for male epigamic and/or behavioral traits of strength. At the same time, physicians in France and the US began to define "hysteria" in women as a long-term complication of sexual frustration that should be treated with manual clitoral and/or vaginal stimulation. In the 1800s, "muscle beaters" used for massage were applied to the clitoris in the treatment of "hysteria". Some of these had long handles that women would use on their own to provide clitoral and vaginal stimulation. In the late 1800s, vibratory stimulation of the clitoris and/or vagina was seen as particularly effective in inducing orgasm, and was easily produced electrically using saline electrodes with faradaic current applied.[8] Thus the "vibrator" was used in clinical medicine explicitly as a means of "electrotherapy" for the treatment of "hysteria". Vibrators were sold by mail order (e.g., from successive catalogs of Sears, Roebuck and Co.) up to the start of World War II as "Aides that Every Woman Appreciates". Although these seemed to disappear in the 1940s and 1950s, they returned in the 1960s specifically as "sex toys" for women. A fascinating account of the development of the vibrator can be found in Maines.[8]

The ovaries had been known since the time of Aristotle to be involved in both the generation of offspring and in female sexual behavior (for a historical overview, see Ref. 9). Despite Aristotle's writings, it was not until 1672 that de Graaf explicitly described the ovarian follicle as an "egg" which turned into a corpora lutea when the female was impregnated. Van Leeuwenhoek in 1683 suggested that it was the egg itself that was impregnated with sperm. In the nineteenth Century, ovarian function was again the focus of scrutiny. The French physician Roberts reported that Indian women who underwent forcible OVX as prepubertal girls had no sex drive, no menstruation, and had retained a boyish appearance (meaning no breasts). Although Berthold in 1849 had suggested the existence of a floating substance secreted by the testes that masculinized body and behavior in roosters, nothing was mentioned about the ovaries until Brown-Séquard's claim in 1890 that multiple injections of guinea pig and rabbit ovarian extracts could refeminize and excite the passions of OVX, hysterectomized women.[1] Berthold's experiment was repeated in 1896 by Knauer, but this time grafting ovaries into the abdominal cavity of OVX dogs, rabbits, and guinea pigs, and restoring estrous cyclicity and sexual behavior.

The early twentieth century saw experiments aimed at discerning the function of the corpora lutea in the timing of ovulation and the maintenance of pregnancy. Heape in 1900 coined the terms estrus, proestrus, diestrus, metestrus, and anestrus to describe cytological changes in vaginal epithelium, which were used subsequently in 1922 by

Long and Evans to link the stage of the vaginal epithelial cycle with sexual ("estrous") behavior. In 1923, Allen and Doisey determined that vaginal cell cornification could be used as a bioassay to determine which of the ovarian secretions induced it. Parkes and Bellerby in 1926 referred to the active secretion, "oestrin", as the cause of cornification, and in 1930 abundant sources of oestrin had been found in the late pregnancy urine of Canadian women and sold by Ayerst Labs as an orally active source of the hormone. In 1929, Butenandt and Doisey determined the crystalline structure of estrogens, and in particular estradiol-17β, which was the same in the late-pregnancy urine of cows, sows, horses, and humans. Progesterone was isolated in 1934 and was found to inhibit pregnancy when injected alone to a variety of gonadally intact, ovulating animals. Work by Zuckerman in 1937 showed that menstruation was the result of atrophy of the corpora lutea. These findings together led to the creation of steroid biochemistry, and ultimately the isolation of different estrogens and progestins, and the formation of oral contraceptives in 1953 by Pincus and Chang.

The other important endocrine question was behavioral. In 1939, Boling and Blandau found that sequential injections of estradiol followed 48 h later by progesterone induced sexual "heat" (lordosis) in OVX female rats. This pivotal paper coincided with experiments throughout the 1930s–1950s by Stone, Ball, Beach, Larsson, Yerkes, and Young, among others, examining the sexual behavior of female rats, cats, and nonhuman primates, such as macaques and chimpanzees, and in particular its expression around the time of ovulation and how it declined after OVX, and was stimulated. An important comparative approach was taken to this, and careful analyses were made comparing the expression of hormone-driven sexual behavior in animals to humans (e.g., Ref. 10). Since the 1930s, oral estrogens derived first from urine, and then made synthetically, were used to treat menopausal symptoms such as hot flashes. With the advent of the birth control pill and its subsequent reformulations, hormone replacement therapy was born. In the late 1960s, binding sites for estradiol were found in the brain independently by Pfaff, Sar, and Stumpf, and localized largely in the mediobasal forebrain, notably in regions of the hypothalamus and limbic system. The molecular actions of those receptors were characterized in the 1980s and 1990s by McEwen, Pfaff, and others, and their molecular role in the generation of neurotransmitter actions was elucidated.

Drugs of abuse, such as heroin and cocaine, had been known since the 1920s to alter reproductive function in both women and men and to inhibit sexual arousal and desire, along with anorgasmia, in female addicts.[11] The effects of alcohol on sexual arousal, desire, and reproductive function were legendary.[12,13] The study of drug effects on sexual behavior began in the 1950s with the work of the Soulairacs in France. The role of the newly discovered monoamines, dopamine (DA) and serotonin, was examined on lordosis in female rats using systemic pharmacological treatments by Swedish pharmacologists Meyersson, Ahlenius, Södersten, and Malmnäs starting in the mid-1960s through the 1970s. The discovery of brain-born neuropeptides and their receptors in the 1970s and 1980s increased the complexity of the pharmacological targets, and individual brain regions, especially in the hypothalamus, began to be examined. Large brain lesion studies in the 1940s and 1950s showed that decorticate male rats could not copulate, but that OVX females receiving estradiol and progesterone could still display lordosis.[14] More specific brain lesion studies conducted largely in rats during the 1960s showed that ablation of the medial preoptic area (mPOA) reduced male mounting behavior[15] and lesions that included the ventromedial hypothalamus (VMH) reduced lordosis in female rats.[16,17]

The analysis of female sexual behavior in different species became more sophisticated during the 1980s and 1990s (see below), as did the pharmacological and molecular tools applied to its study. Transgenic mouse models were made with specific genes deleted (knockouts (KOs)) or overexpressed (knock-ins); antisense technology allowed researchers to KO specific gene products in different brain regions. Microdialysis and voltammetry allowed certain monoamines, such as DA and serotonin (5-HT), and small molecule neurotransmitters such as acetylcholine (Ach), glutamate, and GABA, to be analyzed directly in brain regions. Cellular techniques allowed cytoplasmic proteins or their mRNA to be labeled in brain slices, and the use of immediate-early gene products such as Fos allowed cell-level localization of activated neurons following copulatory stimulation.[18] Similar advances were made in human brain imaging using functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) scanning. These techniques allowed clinical researchers to examine brain activation by erotic stimuli and orgasm in women (see below).

Sophistication also grew in behavioral analyses of sexual function and dysfunction in women, although not without heated controversy. In 1948, Kinsey and colleagues published *Sexual Behavior in the Human Male*,[19] which was greeted with a sense of accomplishment and intrigue in the postwar West. Five years later, their publication of *Sexual Behavior in the Human Female*[20] was greeted with scandal and denouncement. Nevertheless, Kinsey's work, along with the advent of *Playboy* as a mainstream publication, and the reaction a decade later to *Human Sexual Response* by Masters and Johnson,[21] gave rise to a "sexual revolution" during the 1960s and 1970s that had women question traditional sex roles and traditional sexual behavior, especially the role of the clitoris as *the* gland of women's orgasm and sexual pleasure, rather than the "mature" vaginal orgasm that Freud[22] had thrust

into psychoanalytical therapy and psychiatric medicine. Hite[23,24] wrote two reports on male and female sexuality in the sexually liberated 1970s, which saw changing female sexual attitudes. However, she still noted sex differences in which women were viewed as more "sensate focused" and needing more than visual erotic stimuli to arouse their sexual interest. Such sex differences in response to erotic visual stimuli, however, appear to have waned with the advent of the Internet, and with a new generation of women who have had easy access to such stimuli.

Sexual disorders, which had been cataloged by psychiatry in various editions of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) in North America and the *International Classification of Diseases* (ICD) in Europe, eliminated homosexuality as a disorder in the mid-1970s and reworked sexual behavior disorders in women as those of sexual arousal, desire, and orgasm, with sexual pain disorders, such as dyspareunia and vaginismus, and gender identity disorders as separate disorders. Work around the turn of the millienium sought to characterize those disorders as sets of symptoms and apply both physiological and subjective assessment techniques that could predict symptom severity. As with the Kinsey experience a generation before, the advent of oral treatments for erectile dysfunction in men was hailed in light of a new sophisticated neuropharmacology of sex, whereas the search for a "female Viagra" has been vilified by special-interest groups who are against the so-called "medicalization of women's bodies" by the pharmaceutical industry.[25,26] Although androgens were reported to increase sexual arousal and desire in both pre- and postmenopausal women with those disorders, a transdermal patch was rejected by the U.S. Food and Drug Administration. Despite this, more compounds entered clinical trials for the treatment of sexual arousal and desire disorders in the early twenty-first century. Part of that effort was aided by preclinical research in rodents and nonhuman primates, showing conclusively that drugs that elevated female solicitations in rats also increased measures of subjective sexual desire in women. The study of the neuroanatomy and neurochemistry of sexual behavior has become another "sexual revolution" of neurobiologically-relevant information about sex—one that moves almost seamlessly between species. This begins to realize the challenge put forth by Beach[27] to examine the physiology of behavior across different species and the dream of a truly translational clinical science.

COMMONALITIES, HOMOLOGIES, ANALOGIES, AND MODELS

Sexual function is required for the propagation of all mammalian species. It not only allows for reproduction, but it also provides a natural reward that is easily accessible to any sexually mature individual in good health. Its benefits can be short-term by instilling feelings of satiety, intimacy, and well-being, while long-term benefits may include the memory of the sexually satisfying event, bonding with the partner, in addition to the pregnancy and the delivery of offspring. Sexual dysfunction, in contrast, results in feelings of inadequacy and a loss of feelings of intimacy and well-being, which induces significant distress on individuals and their relationships. The etiology of the dysfunction can be due to physiological, psychological, or a mixture of factors. As such, sexual function in humans involves a complex integration of the individual's biology and physiology (including genetics, hormonal states, and neurochemical regulation), personal history, expectations, context, and culture—all of which impinge on the brain to create or inhibit a sexual response.

In all species, sexual behavior is directed by a complex interplay between steroid hormone actions in the brain that give rise to sexual arousability, and experience with sexual reward or pleasure that gives rise to expectations of competent sexual activity, including sexual arousal, desire, and elements of copulatory performance.[28] Sexual experience allows animals to form instrumental and Pavlovian associations that predict sexual outcomes and thereby direct the strength of sexual responding. Although the study of animal sexual behavior by neuroendocrinologists has traditionally been concerned with mechanisms of copulatory responding related to reproduction (e.g., lordosis in females and erections, mounts, intromissions, and ejaculations in males), more recent use of conditioning and preference paradigms, and a focus on environmental circumstances and experience, has revealed sexual behaviors in a variety of species that are driven by reward-related mechanisms in the brain and that are analogous or homologous to human sexual desire.[28–31] From both a biological and psychological perspective, this makes logical sense: animals must be able to respond to hormonal and neurochemical changes that signal their own sexual arousal and desire, and be able to interact with external sexual incentives. Animals must be able to identify external stimuli that predict where potential sex partners can be found and subsequently seek them out, solicit, court, or otherwise work to obtain them; distinguish sensory cues and behavioral patterns of potential partners from those that are not interested or receptive; and pursue desired sex partners once sexual contact has been made.

The Sexual Brain

The brain organizes sexual stimulation into an evolutionarily conserved set of pathways or "modules" (e.g., Ref. 32; see below for lordosis) that reflect different levels of processing and interpretation[33] (Figure 50.1).

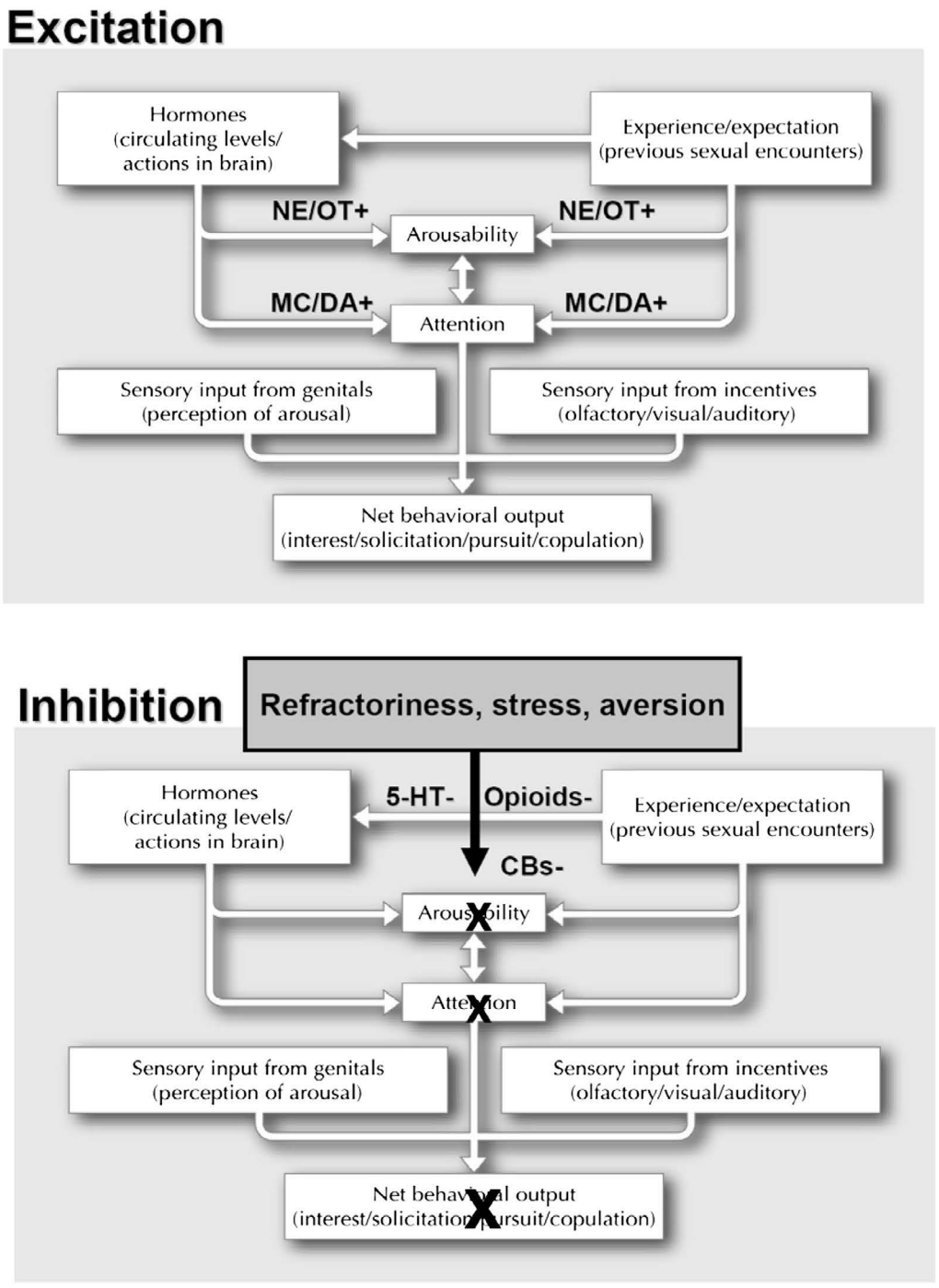

FIGURE 50.1 Interactive model depicting the regulation of sexual behavior by hormonal and associative learning (experiential) systems that subserve sexual arousal, desire, and behavior. Top: Excitation produced by hormone action and/or experientially derived activation of arousability (via activation of norepinephrine (NE) and oxytocin (OT)) and attention (via the activation of melanocortin (MC) and dopamine (DA)) that mixes with peripheral arousal and sexual stimuli to drive net behavioral output. Bottom: Inhibition from refractory states, stress, or aversion. Inhibitory systems activate serotonin (5-HT), opioids, and endocannabinoids (CBs) to induce satiety, pleasure, and sedation, respectively, although such systems are activated in stressful or aversive circumstances. *Source: Adapted from Pfaus and Scepkowski.*[33]

These pathways integrate endogenous sex "drive" (e.g., gonadal hormone status and energy metabolism) with autonomic arousal in the hypothalamus, the intensity of incentive sexual stimuli (unconditioned and conditioned stimuli that activate or "prime" attention and movement from distal to proximal to interactive) in the hypothalamus and limbic system, and the evaluation of sexual context and executive function as it relates to sexual excitation or inhibition overall in the cortex. In particular, cortical activation controls the coding of information

into "gestalts" (e.g., sets of physical or interpersonal characteristics that individuals find conditionally attractive or unattractive, contexts that are suitable or unsuitable for sexual activity, etc., following from Pavlov[34]) and involves the activation of medial prefrontal cortex (mPFC) and the descending inhibition of motor acts as part of executive function. Within each are excitatory and inhibitory neurochemical systems that control sexual responding at any given time. These systems are activated or suppressed by steroid hormones, as well as by experience-driven changes in gene expression and neurochemical function.[35] It is through these systems that priming stimuli or drugs alter sexual responding by changing the interpretation of stimuli and context.

Attentional and emotional components are encoded largely in limbic structures, notably in the nucleus accumbens (NAc), septum, and amygdala, which allow the animal to focus on pleasure- (or punishment-) related stimuli in the environment. The hippocampus provides spatial maps of the external world and episodic memory for important sexual encounters, and the paleocortex (e.g., anterior cingulate gyrus) regulates autonomic function along with anticipation of reward, decision-making, and empathy.[36,37] Along with limbic activation, hypothalamic structures, notably the mPOA and VMH, activate sexual responding in relation to hormonal status and metabolism, and in concert with regions, such as the paraventricular nucleus (PVN) and supraoptic nucleus (SON), coordinate autonomic activation with elements of sexual desire (e.g., solicitations, pursuit). Those structures also participate in the generation of partner and mate preferences. The mPOA is well suited as a central processor in the linking of metabolic need, hormonal status, and autonomic outflow, with the stimulation of mesolimbic DA neurons in the ventral tegmental area (VTA). The mesolimbic DA system projects to several important limbic and cortical structures, notably the NAc, corticomedial amygdala, lateral and medial septum, and mPFC, and is critical for all animals' attention to incentive stimuli.[38] Thus, regulatory, attentional, and emotional systems are engaged at the same time following the hormonal stimulation that occurs around ovulation, linking reward-related incentive motivation to reproduction.

Finally, although sexual responses can include thoughts and fantasies (at least in humans), they are reflected in all animals as behavior. Coordinated purposeful behavior comes from the activity of both fine and gross motor acts that are derived from the coordinated activation of motor cortex and the basal ganglia, along with other motor structures in the midbrain and the cerebellum. In addition to coordinating body movements in space and time, these structures crystallize motor memory, a function that is critical for motor habit formation (the phenomenon whereby motor acts at the beginning of behavioral learning are choppy and uncoordinated, but become virtually automated with practice). Although the formation of motor habits in males with extensive sexual experience protects sexual behavior against treatments or situations that might disrupt it, including novel environments, stress, genital anesthesia, brain lesions, and even castration or hypogonadism (reviewed in Pfaus et al.[28]), it is not yet known whether sexual experience provides females with similar protection.

Structure of Female Sexual Behavior

For all animals, sexual behavior occurs as a sequence or "cascade" of behavioral events. Beach[39] recognized the heuristic value of separating sexual behavior into *appetitive* and *consummatory* phases. Essentially, this scheme followed from the work of early twentieth century ethologists and experimental psychologists,[40,41] who defined appetitive (or "preparatory") behaviors as those which bring an animal from distal to proximal and into contact with goal objects or incentives, such as potential sex partners. In contrast, consummatory behaviors are performed once an animal is in direct contact with the incentive (i.e., to "consummate" the goal). Consummatory sexual behaviors tend to be species-specific, sexually differentiated, and stereotyped, whereas appetitive behaviors are more flexible. This also makes sense as survival often depends on behavioral flexibility—on an animal's ability to learn a variety of strategies to obtain goals in different environments or appetitive circumstances.[28,42] As in animals, human sexual desire and subjective sexual arousal fit into an appetitive framework,[28,31,43] whereas the more stereotyped patterns of copulatory behavior fit into a consummatory framework. Perhaps the most well-known description of human sexual response is that of Masters and Johnson's "EPOR" (Excitement-Plateau-Orgasm-Resolution) model[21] (Figure 50.2). This model flows in time as a cascade of behavioral and neurophysiological events, starting with sexual excitement (blood flow to the genitals and other erogenous erectile tissues), then plateau (parasympathetic maintenance of genital blood flow during sexual intercourse), culminating in orgasm (a defining moment of euphoria, ecstasy, and pleasure in which sympathetic systems move blood out of the genitals), followed by resolution (also called a refractory period during which inhibitory systems of the brain are activated to reduce the salience of external and somatosensory sexual stimuli). The EPOR model describes at least three distinct patterns for women that vary in the structure of the plateau, the intensity and number of orgasms, and the temporal offset of arousal during the resolution phase, although it does not differentiate the particular characteristics of the sexual stimuli used to achieve orgasm (e.g., external clitoral

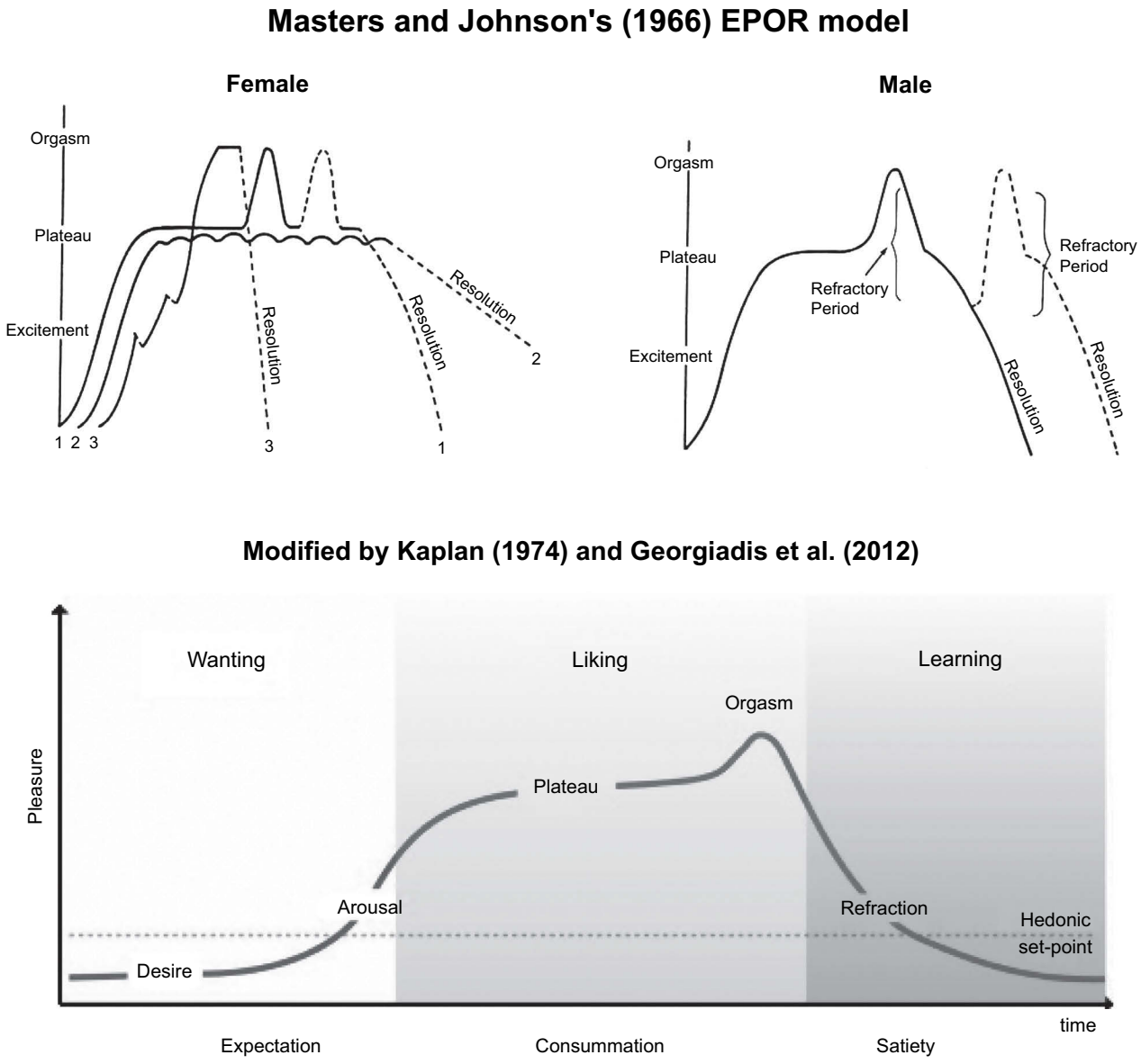

FIGURE 50.2 Top: The EPOR model of human sexual response by Masters and Johnson.[21] Bottom: Modifications of the model by Kaplan[44] to include sexual desire before arousal, and further modifications by Georgiadis et al.[45] to include theoretical phases of expectation, consummation, and satiety, along with the conceptual framework of Berridge et al.[46] of wanting, liking, and learning.

only, external and internal clitoral, cervical, blended clitoral and cervical, extragenital, etc.), nor was it based on an analysis of actual genital blood flow. Subsequently, Kaplan[44] added a phase of sexual desire, consisting of fantasies and thoughts about sexual activity, along with behavior aimed at obtaining sexual partners and/or sexual gratification.

Despite overarching theoretical models of human and animal sexual response that did not posit sex differences in the basic response structure, female sexual behavior has, until fairly recently, been considered "passive". This is due in part to a general social construction in Western society of female sexuality as something that is "done to", relative to more active male sexuality that "performs", and to the labeling of female sexual behavior in both animals and humans as "receptive", consisting largely in animals of estrogen- and progestin-dependent behaviors that allow females to accept male initiation (e.g., mounts) and be open to vaginal penetration by engaging in postural changes such as lordosis, the characteristic arching of the back that raises the rump to allow penile intromission. Similarly, in humans, hormone- and context-dependent "responsive desire" has been viewed as allowing females to be responsive to a partner's active pursuits.[47] However, it is clear that women and some other primate females can have sexual intercourse anytime during the ovulatory cycle. This can even occur without hormone priming in hypogonadal individuals and, indeed, without

prior desire or consent.[48] Although sexually receptive behaviors clearly exist in females of all species, they are far from passive when it comes to sex. Based on observations of a variety of species, Beach[39] proposed that female-initiated sexual behaviors can be partitioned into a cascade of essentially three temporal phases: *attractivity* (behaviors such as approach or scent marking that lure males to the females), *proceptivity* (behaviors that precede receptive behaviors and focus the male on pursuing the female), and *receptivity* (behaviors such as lordosis and lateral tail deflection in rats and hamsters, respectively, or leg spreading in the human) that allows the male to gain vaginal penetration. More recently, Basson[47] (Figure 50.3) described how innate sexual desire (potentially induced at ovulation, for example by the combined action of estrogens and androgens in the hypothalamus and limbic system) activates attention to incentive sexual stimuli, sexual arousal, and sexual receptive behaviors, that, if positively reinforced, lead to a sensitization of attention and sexual arousal in the presence of salient and competent incentive sexual cues. Her model is easily applicable to all species and is similar to incentive models for sexual motivation produced by others.[2,43] Inherent in all models of sexual behavior is the notion that the components are separable. This would require different brain regions or networks to control the components, feedback systems that link them together, and molecular mechanisms that allow their activation to be altered by steroid hormones and experience.

Clearly, females and males engage in mutual and complementary patterns of sexual activity; however, it is the females that initiate and control successful sexual interaction, including the initiation and temporal patterning of copulation. This occurs by a complex interaction of *appetitive precopulatory behaviors* that attract and solicit sex from males. These behaviors are taken to reflect sexual desire and may well be informed by or sum with sexual arousal. Once copulation begins, females engage in *receptive behaviors* such as lordosis, *pacing behaviors* that control the rate of sexual stimulation received during sexual interaction and copulation, and *defensive behaviors* used either to pace the copulatory contact if females cannot otherwise do so or to terminate the sexual interaction.[2,49,50] These behaviors serve to optimize the rate and strength of sexual stimulation received by females, which in turn initiates neuroendocrine reflexes associated with fertility and pregnancy.[51–53]

Ovarian Hormones Set the Stage

The cyclic actions of estradiol, testosterone, and progesterone in females leads to changes in sexual responding and increases in sexual arousal and desire around the time of ovulation in all vertebrate species, including humans,[54,55] although a smaller increase in arousal and desire has been reported around the time of menstruation.[56] Of the major steroids released from the ovaries of mammalian females, estradiol and testosterone are at their highest level in the circulation around the time of ovulation (Figure 50.4). Progesterone levels rise before, during, and after ovulation, depending on the species. This hormonal milieu during the periovulatory follicular phase alters the way in which visual sexual stimuli are processed in women,[58–61] which presumably leads to a shift in the incentive value of the stimuli. Analogous findings have been reported in other primates, for example, in approaches and solicitations made around the time of the mid-cycle estradiol peak in rhesus macaques,[57] and in the appetitive and consummatory sexual behaviors that characterize the periovulatory period of female rats.[31,32,39,49,50,62] Steroid hormones drive sexual arousal

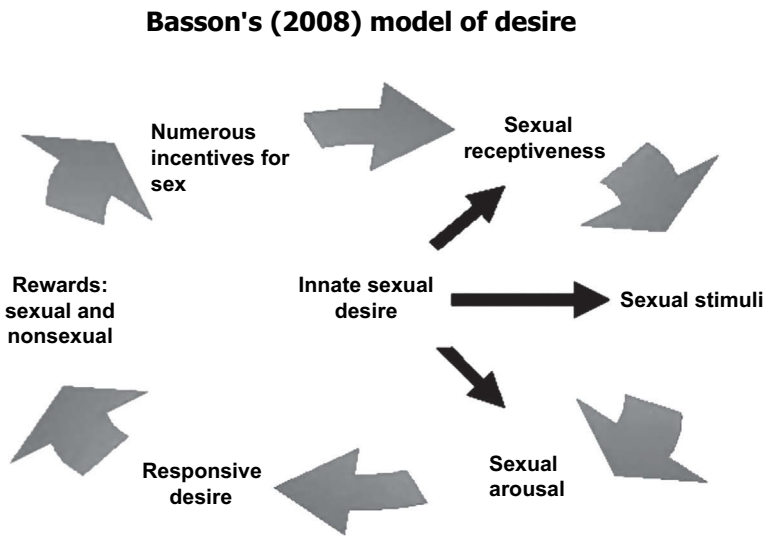

FIGURE 50.3 Circular model of female sexual response by Basson.[47]

and desire in response to competent incentive stimuli. In turn, experience with sexual reward (and inhibition) modulates the strength and trajectory of responses to incentive sexual cues. This is timed in most female mammals to the period around ovulation, thus stimulating females to engage in the most rewarding behaviors under the most reproductively relevant circumstances (see below). This contrasts with the relatively stable and continuous testicular androgen secretion in mammalian males (and its aromatization to neural estradiol in different regions of the brain) that maintains sexual arousability and responsiveness in a relatively continuous manner[63] (see Chapter 49).

Animal Models of Female Sexual Behavior

Although human sexual behavior is best studied in humans, it is often impossible to do so with experimental precision or at a level that allows any degree of finely-grained neural or molecular analysis. Recent advances in brain imaging and eye-tracking technology have allowed cortical and subcortical activation and visual gaze to be assessed in women viewing erotic visual stimuli, and some important paradigms have emerged to correlate aspects of subjective sexual arousal, desire, and orgasm to overall brain activation patterns.[64] Such data reveal a great deal about the cognitive and limbic control of different aspects of female sexual behavior under different hormonally-modulated, pharmacological, or experiential conditions, and in ways that confirm data from females of other species. Nevertheless, these paradigms lag behind the scope of neuroanatomical, neuropharmacological, histochemical, and molecular methods that can be utilized in situ with animal models.

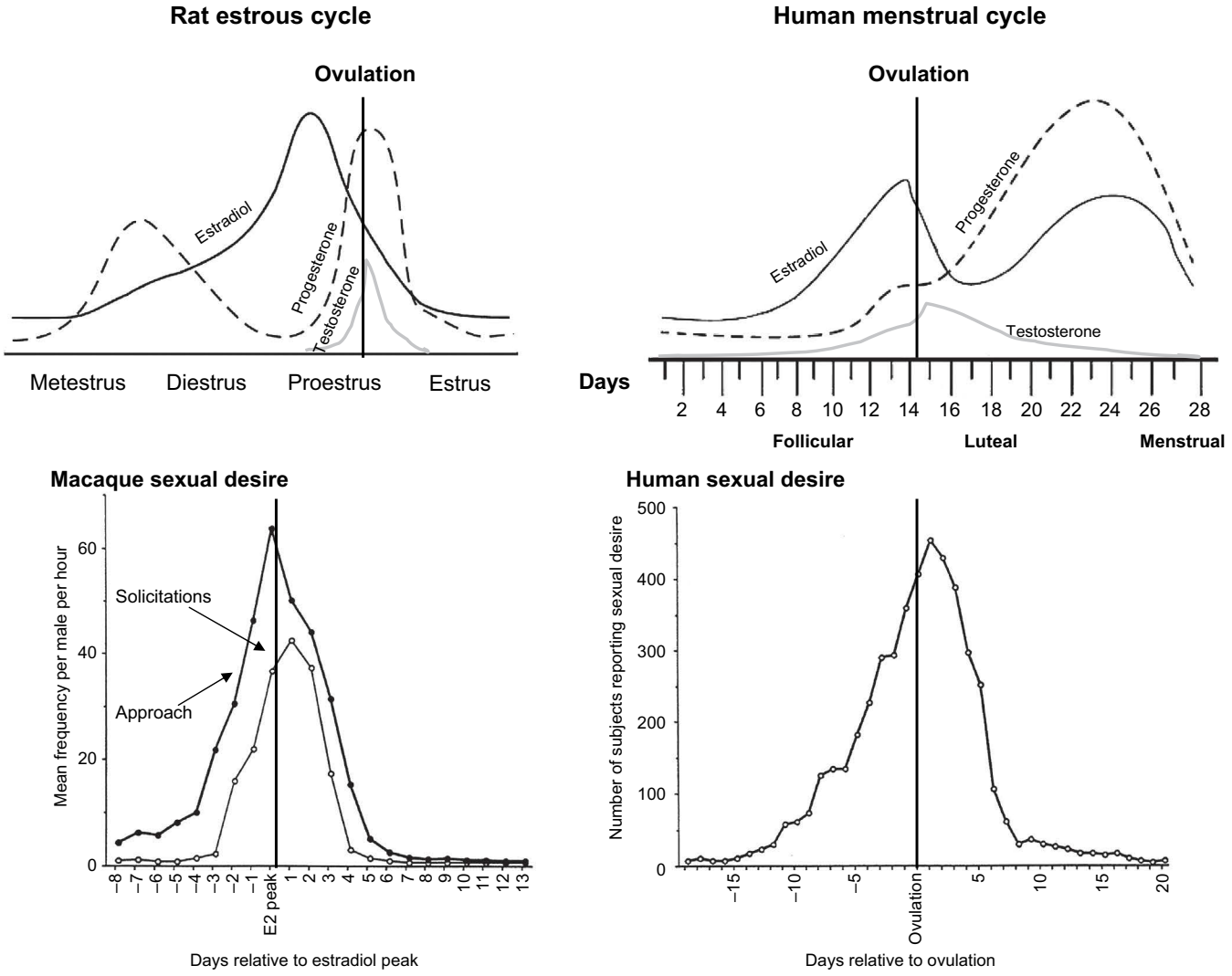

FIGURE 50.4 Top: Schematic changes in estradiol, progesterone, and testosterone in rats and humans across their respective estrous and menstrual cycles. Bottom: Sexual approaches and solicitations by female rhesus macaques, and expressed sexual desire of women, relative to ovulation. *Source: Adapted from Refs 54,57.*

Most of the research done on the neurobiology of female sexual behavior comes from rodent subfamilies, such as rats, mice, hamsters, gerbils, voles, and musk shrews, lagomorphs like rabbits and myomorphs like guinea pigs, less so from primates like rhesus and Japanese macaques, and even less so from humans. Understanding the behavioral structure of each species' appetitive and consummatory phases is vitally important for a sophisticated understanding of the neurobiological mechanisms that control it. In many studies the female's receptive lordosis posture is taken as an index of her copulatory or "mating" behavior, rather than the full repertoire of appetitive and consummatory sexual responses. It is often the case in laboratory settings that females are tested in small chambers that do not allow them to approach or escape the male or, in the case of females that pace the copulatory contact, allow them to regulate the timing and intensity of that contact. When appetitive responses are taken together with lordosis, it becomes immediately apparent that there is extensive conservation of the neurochemical mechanisms that control sexual behavior which generates homologies in the way that sexual stimulation is perceived by the brain and induces competent responses. Behavior is the ultimate arbiter, and can never be supplanted by the processes that underlie it. For example, release of the neurotransmitter DA in mesolimbic terminals like the NAc may be involved in all forms of appetitive motivation toward rewarding incentives like sex partners, but just observing DA released there does not allow the viewer to conclude that whatever the animal was doing is positively hedonic. Understanding the neurobiology of animal sexual behavior also allows the consideration of models of sexual function and dysfunction that are directly applicable in the preclinical testing of drugs or other treatments.

Rats

Female rats are cyclic ovulators whose sexual behavior is timed to the periovulatory period. The ovulatory cycle last approximately 4–5 days, and as mentioned above, is split into phases that reflect the actions of ovarian hormones on vaginal cell morphology and sexual behavior, including diestrus, metestrus, proestrus, and estrus. A rise in estradiol, during metestrus, followed by a peak in progesterone during the afternoon of proestrus, just prior to ovulation, primes the neural circuits necessary for sexual responding. As such, sexual behavior is expressed only during an approximate 12–20 h window, when she is fertile. As mentioned above, sexual behavior can be completely eliminated by bilateral OVX, but reinstated by priming with estradiol and progesterone (P) administered by subcutaneous injections, 48 and 4 h prior to testing, respectively,[65] to mimic ovarian steroid secretion.

A typical copulatory bout in rats begins by anogenital investigation by either the female or the male, followed shortly thereafter by a solicitation made by the female. Solicitations begin with a head-wise orientation toward the male followed by a runaway of varying lengths, some of which are short and in the male's vicinity and are typically referred to as "hops and darts".[30,31,49,50,66–71] This behavior entices the male to chase the female. As the female comes to a stop, she initiates a present posture, facilitating the ability of the male to mount the female when he arrives. Flank stimulation during a mount elicits lordosis, the concave arching of the back that raises the rump and anogenital region, and is absolutely critical for vaginal penetration, or intromission, to occur. Male mounts are almost always accompanied by pelvic thrusts which make direct contact with the female's clitoris. If the male has an erection, then the pelvic thrusts will push his penis into her vagina. Such vaginal intromissions provide the female with stimulation of the vagina, including the internal clitoris and possibly also the cervix. Immediately after a vaginal intromission the male dismounts and grooms his penis into detumescence. The female may run away again or stay in the male's vicinity and hop over him to induce another mount with intromission. In this way, the female regulates or "paces" the rate of vaginal penetration, which controls the rate of vaginal, clitoral, and possibly cervical stimulation. After several mounts with intromission regulated by the female, the male ejaculates. Ejaculation consists of a deep penetration that is held in the vagina as the ejaculate congeals into a "vaginal plug" that surrounds the cervix and protects sperm transport. This also produces a large and sustained amount of cervical stimulation and is responded to by the female with a bursting dismount and runaway. After a few minutes of absolute then relative refractoriness and behavioral quiescence, the female shows increasing interest in the male and stays in his vicinity. This is responded to by the male with the resumption of mounts with intromission and a second ejaculatory series. Males typically have 7–10 ejaculatory series before becoming "sexually exhausted".[72,73] However, long before male exhaustion, females display a progressive reduction in solicitations and enforce increased time between intromissions either by running away or fighting. In the wild, more dominant females tend to reenter the burrow after only a few ejaculations, whereas more subordinate females take more ejaculations before they stop copulating. The progressive decline in solicitations and increase in agonistic behavior is characteristic of "estrus termination", an inhibitory state that signals a period of female refractoriness and the endocrine transition to pseudopregnancy or pregnancy.[70,74,75]

Female and male rats have also been used in studies of sexual reward, and to understand the plasticity of appetitive responses and their neurochemical control under different sexual circumstances, including different contexts, experiences, level of sexual reward, and partner

density.[28,30,76–79] Indeed, rats will copulate in a variety of circumstances and testing chambers, including small cylindrical or square chambers,[80] unilevel pacing chambers,[49,77,78,81–84] bilevel pacing chambers,[70,75,85,86] and open fields[31,50,87] (Figure 50.5).

Mice

There are well over 1100 naturally-occurring species of mice, with a far larger number of transgenic lines made with specific gene deletions or insertions. Those lines have been useful in identifying the role of estrogen, androgen, and progestin receptors using lines with specific KOs of those receptors or their subtypes, e.g., estrogen receptor alpha (ERα) and ER beta (ERβ), or steroid synthesizing enzymes, e.g., aromatase or 5α-reductase. The sexual behavior of wild house mice (*Mus musculus*) was documented by Estep, Lanier, and Dewsbury,[87] and consisted of a period of appetitive courtship that included mutual grooming of the body and genitals, sniffing of the genitals, and rooting (in which one partner lifts up the body of the other with its head). Females were observed to orient away from the male prior to the males initiating mounts. Like hamster females, these wild mice hold lordosis for a long duration of male thrusts with intromission. Upon ejaculation, males typically fall over, pulling the females with them. Females then hold lordosis on their sides with the male's penis still inside the vagina for approximately 20–30 s, after which the male dismounts, and the two groom their own genitals. Unlike the female rat, the female mouse stays in close proximity to the male during his postejaculatory interval. Despite obvious differences in copulatory behavior

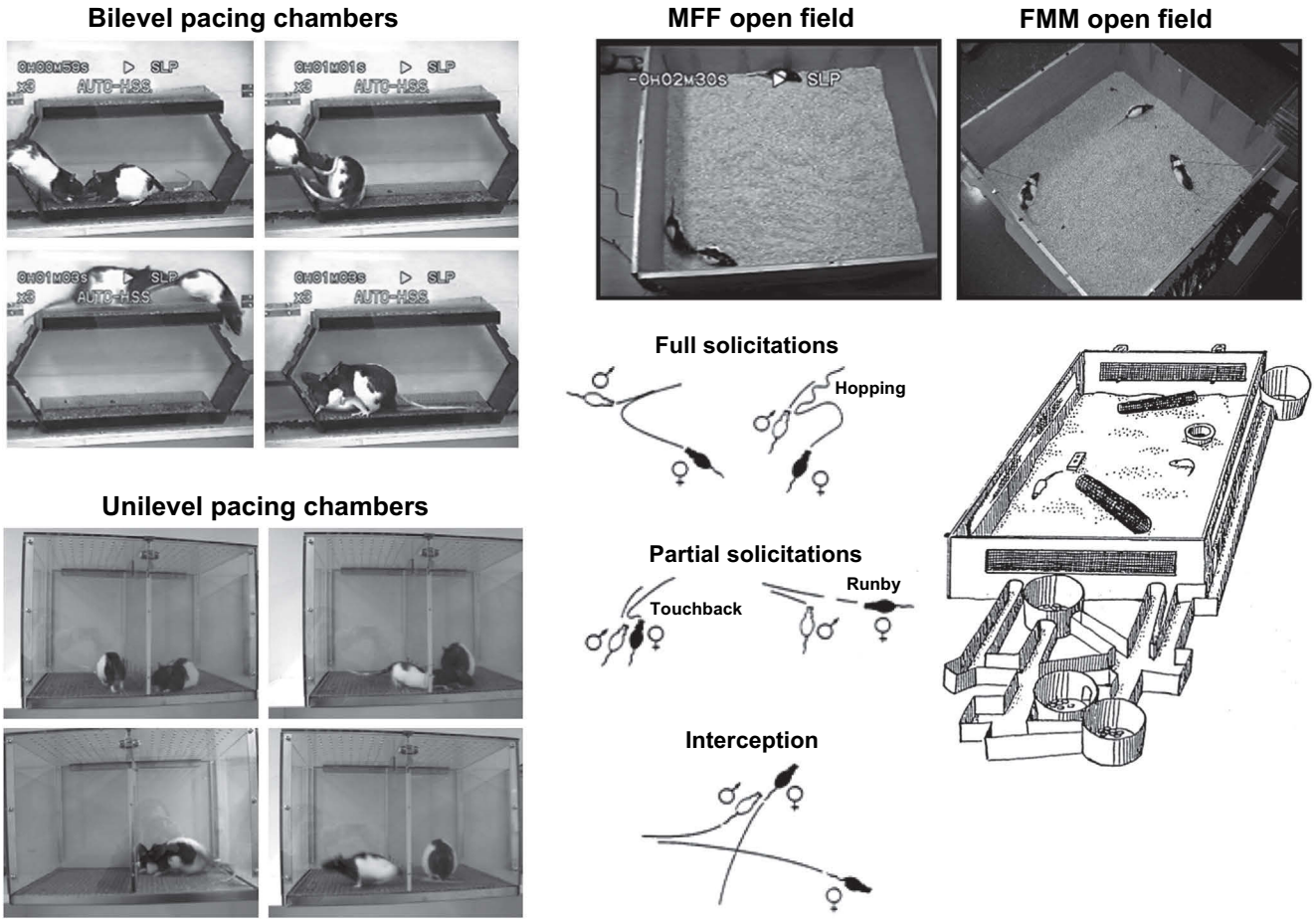

FIGURE 50.5 Rat copulation in several chambers. Top left: Bilevel pacing chambers. Bottom left: Unilevel pacing chambers. Bouts of copulation are typically initiated by the female through solicitations. In bilevel chambers the female solicits as she would in the wild by making a headwise orientation to the male and then either darting or hopping away on the same or other level. This forces the male to chase her and females regulate the rate of copulation by running from level to level. The endpoint of each solicitation and runaway is lordosis, allowing the male to mount and gain vaginal intromission, after which the male dismounts and grooms his penis. This is repeated several times until the male ejaculates. Unilevel pacing chambers are bisected by a Plexiglas or mesh partition with one or more holes cut out of the bottom that are big enough to allow the female to pass through but too small for the male. This restricts the male to one side. Females initiate copulatory contact by moving to the male's side, and regulate the rate of contact by running from side to side. Top right: Open fields used for tests of conditioned sexual partner preference for one male choosing among two free-ranging females (MFF) or for one female choosing between two tethered males (FMM). Bottom right: Large open field used by McClintock[50] to examine group mating patterns and preferences in female rats.

between mice and rats, nearly identical pacing behavior was observed recently in female mice.[88] Females placed into a smaller version of a unilevel pacing chamber bisected by Plexiglas dividers with four holes that only the females can pass through displayed exits and returns from the male's side after bouts of intromissions. This indicates that mice pace the copulatory contact like rats do when given the opportunity. It is not yet known whether female mice find sexual stimulation in paced conditions rewarding.

Guinea Pigs

Guinea pigs were used extensively to understand the role of ovarian hormones in the elicitation of lordosis, and also to understand the functions of progesterone. Both females and males engage in a courtship dance prior to copulation in which the female approaches the male swinging her hips back and forth and making a vibrating sound often referred to as "purring". Males respond in kind, and will often sniff and lick the anogenital region of the female, who does the same. Males have been observed to purr and sway their hips at nonreceptive females, but if the females do not respond sexually, then copulation is not attempted by the male.[89] This is similar to sexually experienced male rats that investigate sexually nonreceptive females but do not attempt copulation.[90] Females in heat express lordosis in response to male flank palpation during mounting. Like rats, males have a multiple intromission pattern prior to ejaculation and, as in rat pacing behavior, females run away from the males between intromissions causing the males to chase them. As the period of estrus terminates, females display more and more aggressive rejection responses to male pursuits and mounts.

Hamsters and Gerbils

The copulatory behavior of these subfamilies have been studied in less detail than that of rats, and mostly for effects of hormones, drugs, or brain region lesions on lordosis,[91] but also to examine social conditions like group size on sexual receptivity,[92] and sexual recognition.[93] Like rats, hamsters become sexually receptive every 4 days during their ovulatory phase, but are much more consistent than rats in their estrous cycles. Gerbils are sexually receptive approximately every 6 days. Female hamsters and gerbils both display appetitive responses that attract and solicit males to chase them. Female gerbils, for example, display a rhythmic "thumping" of the hindlegs that attracts males to them (males display similar behaviors with other males, although typically in response to a threat). Females of both species also display lordosis upon somatosensory stimulation of the flanks and perineum by males. Although both species are seasonal breeders in the wild, sexual behavior can be induced at regular intervals by injections of estradiol and progesterone, as in the rat. True receptivity to intromissions in the hamster, however, is not assessed by lordosis alone. Female hamsters display the lordosis posture and remain in it while the male mounts, intromits, and dismounts several times. To allow vaginal penetration, the females' tails must deflect laterally or dorsally, making "tail displacements" the true measure of receptivity to vaginal penetration.[94] In addition, female hamsters do not run away from males, or if they do it is for a very short distance and still while in a semi-lordosis posture, so there is little chasing behavior. Males simply mount and intromit repeatedly until ejaculation. Like rats, female hamsters will attack males that attempt to mount them when they are not sexually receptive, when the period of sexual receptivity is terminating, and when they are OVX and primed with low doses of estradiol, with or without progesterone. Female gerbils engage in appetitive thumping, causing males to thump back, as the rhythmic leg thumps are a form of social communication. This typically occurs prior to the female presenting to the male, which is followed by the male mounting. Estrus termination in both species is induced by vaginocervical stimulation (VCS) and accompanied by an increase in female fighting if males persist in attempting to mount. It is more typical, however, for females to take themselves out of the situation by moving to a different space or into a burrow system.

Voles

Voles have been studied largely to understand the neurochemical and genetic basis of "monogamous" vs "promiscuous" sexual partner and mate preferences. Both female and male prairie voles show social and sexual partner preferences for their first sexual partner, relative to an unfamiliar partner, and they form relatively stable pair bonds for the nurturing of their pups. In contrast, closely related meadow voles do not display such preferences. However, prairie voles are not sexually exclusive and will copulate with other partners under certain circumstances. Differences in mating strategy have been linked to the differential actions of oxytocin (OT) in females and vasopressin in males,[95] in particular in males to the greater expression of the vasopressin 1a (V1a) receptor in the ventral pallidum, a motor structure that receives input from the NAc. DA is also important in driving the partner preference in the presence of familiar—and presumably olfactory—incentive cues.[96]

Female voles make precopulatory scent marks when they are sexually receptive, and they will spend more time near male scent marks. Females also approach males and both engage in side-by-side contact, sometimes referred to as cuddling. Males mount females who display lordosis, and although males appear to set the pace, females copulate with tethered males, suggesting that females can initiate copulation. Vole mating in the wild takes place over

several days, during which males ejaculate several times with either one (monogamous) or several (polygamous) females. Like rats, male voles display a multiple intromission pattern prior to ejaculation, suggesting that females receive clitoral stimulation (CLS) and VCS at a particular rate. It is not known which stimuli are responsible for terminating sexual receptivity in the vole.

Musk Shrews

Musk shrews are a primitive eutherian mammal. Female shrews are induced or reflex ovulators that require behavioral stimulation to ovulate and induce increases in circulating estradiol and progesterone. The torturous process of a male "taming" a female shrew into sexual receptivity has been known for a very long time. Indeed, Shakespeare's 1592 play of the same name is a comic analogy about human heterosexual marriage. Rissman and colleagues studied the copulatory behavior of musk shrews extensively in order to make comparative analyses of hormone-brain interactions. Their work established clearly that female musk shrews display a period of intense appetitive aggression toward males which includes biting and scratching, and if the females live in large natal groups, the aggression against potential suitor males sometimes results in death.[97] Virgin females attack males more frequently than sexually experienced females, and exposure to male olfactory cues reduce aggression in virgin females.[98] The most persistent male will eventually gain access to her flanks and mount, which induces tail-wagging, a first sign of sexual interest, followed by a lordosis posture thus rendering her sexually receptive to his intromissions. Testosterone produced by the ovaries and adrenal glands is the most abundant circulating steroid at the time the males begin their approach,[99] and may explain the increased female aggression during this appetitive period. Indeed, crystalline implants of testosterone in the mPOA or VMH of OVX shrews were more likely to induce a full complement of sexual behaviors compared to implants to the bed nucleus of the stria terminalis (BNST), whereas implants of estradiol in the mPOA or VMH induced lordosis and immobility, and reduced the time taken for females to become receptive. This suggests that aromatization of circulating testosterone into estradiol is necessary for the switching from aggression to sexual receptivity. High levels of cortisol are also observed during the induction of sexual receptivity, and blocking cortisol production reduces sexual receptivity.[100] As with rats, extended periods of sexual receptivity also facilitate the induction of pregnancy.[98] It is not known what type of sexual stimulation may contribute to this.

Rabbits

As with rats and guinea pigs, rabbits have been used to examine a host of female reproductive functions, from hormone and brain lesion effects on sexual behavior and scent marking to sexually dimorphic development of the olfactory system (e.g., Refs 101,102). Female rabbits typically approach males and stay motionless beside them. The two gradually rub noses and nibble at each other's fur. If receptive females are eating when a male approaches, he may rush past them and then gradually get closer, often circling stiff-legged with his rump and tail raised high in the air. If the female ignores the male's advances, he often scent marks her with urine. Once the male mounts and intromits, he typically bites the back of the female's neck. After a few thrusts he ejaculates, and like the rat often falls off the side of the female and remains motionless for a while. As the male approaches exhaustion and the female approaches estrus termination, the two start nibbling more and more at a food source.

Macaques and Other Nonhuman Primates

Rhesus macaques have been used in a variety of ways in sex and neuroendocrine research, notably like rats in the study of hormonal and neurochemical systems underlying appetitive and consummatory sexual behavior, but also to study the social conditions under which very different types of sexual responding are induced. Female rhesus macaques living in large natal groups approach males early in their appetitive phase, several days before their estradiol peak, followed by solicitations of males more closely linked to the estradiol peak.[57,103] Solicitations or "invitations" include characteristic "hand reach", "head-duck", and "head-bob" behaviors in the vicinity of the male.[104] Females also assume a lordosis posture when mounted, and males typically mount with a number of intromissions and pelvic thrusts, sometimes occurring in bouts prior to ejaculation. Females display a characteristic clutching reaction after ejaculation in which the female reaches back and clutches the face of the male.[105] These behaviors cease soon after copulation terminates, as plasma estradiol levels decrease. Like rats and dogs, female rhesus macaques also mount sexually inactive or naïve males as a supersolicitational behavior to induce the males to mount back.[106] In contrast to females living in large groups, single female rhesus macaques living in dyadic conditions with one male show a far greater propensity to submit to mounts on the part of the male throughout the ovulatory cycle, although there is an increase in successful mounts by the male around the time of the estradiol peak.[103] Thus, a dyadic context in which the female is always in the vicinity of the male and cannot escape stimulates more mounting behavior on the part of the male, which is reacted to by consummatory sexual behavior on the part of the female throughout her ovulatory cycle, although the number of male ejaculations still increases during ovulation. This suggests that contextual

cues and sexual stimulation interact with neuroendocrine systems to stimulate sexual responding in female rhesus macaques that appears to be less tightly-linked to ovulation, a situation generally not found in rats and mice. In laboratory settings, female rhesus macaques can also be trained to bar-press to obtain a male, behavior that increases during their ovulatory phase.[107]

Female Japanese macaques, and to a lesser extent Stump-tail macaques, engage in homosexual mounting of other receptive females, often to the point of displaying ejaculation—like facial grimaces prior to dismounting and a period of rest,[108] suggesting that female–female mounting leads to genital stimulation that induces an ejaculation-like state in the female. Female Japanese macaques form stable homosexual consortships during the breeding season[109–112] that is not due to, nor affected by, the availability of males. Females mount one another repeatedly, and males entering into this consortship engage in intrasexual competition, often trying to fight the dominant (mounting) female to gain access to the other female, who typically rejects him over 90% of the time.[109] Female bonobos also engage in female–female mounting, usually as a method of conflict resolution when males are fighting.[113,114] Notably this behavior can occur at anytime during the ovulatory cycle. Although the patterns of female approach and solicitation are similar in many primate species, the copulatory stance of the Bonobos includes face-to-face, male-on-top "missionary" positions not observed in other primates except humans.

Humans

Like other primates, the sexual behavior, and especially sexual arousal, desire, orgasm, and sexual inhibition of the human female are exquisitely sensitive to context and social learning.[47] However, despite the general view that sexual behavior in women is "freed" from the dependence on steroid hormones, women display a characteristic increase in self-reported sexual desire and arousal during ovulation.[55] Across the ovarian cycle of women, steroid hormone levels fluctuate in a cyclical manner. Circulating levels of estradiol, progesterone, and testosterone rise around the time of ovulation, correlating with an increase in sexual interest, activity and fantasies.[55,115–118] Removal of cyclic steroid hormone release by long-term administration of estrogen-containing oral contraceptives often results in a decline in sexual desire, activity, and genital blood flow.[115,119,120] It is unclear whether the blunting of the cyclical induction of sexual activity and fantasies is directly due to the removal of the cyclicity of the hormones acting on relevant tissues, or whether it is secondary to downstream effects of chronic administration of estrogens. Long-term exposure to estrogens has a number of physiological effects that might disrupt sexual behavior. For example, estrogens upregulate steroid hormone binding globulin (SHBG) production by the liver, which are transport proteins that bind androgens with higher affinity than estrogens.[121,122] A recent study on premenopausal women's steroid hormone levels using liquid chromatography-tandem mass spectrometry revealed that serum testosterone, free testosterone, estradiol, estrone, and SHBG levels peaked at midcycle and remained higher in the mid-luteal phase, whereas the 5-α reduced androgen metabolite dihydrotestosterone (DHT) did not change across the cycle.[123] Chronic use of oral contraceptives containing combined estrogens and progestins lowers free testosterone levels and upregulates SHBGs.[124] Given the importance of free testosterone for sexual desire in women,[125] its reduction by chronic oral contraceptive use may be one reason why some women experience a decrease in sexual desire on the pill.

A decline in sexual desire and activity also occurs following surgical and natural menopause. Surgically menopausal women, induced either by bilateral oophorectomy and hysterectomy, experience a sudden and drastic decline in sexual arousal and desire.[126–129] These symptoms can be restored following adequate hormone replacement regimens, particularly with replacement of estrogens in combination with testosterone.[126,127,130–139] As such, ample evidence suggests that fluctuating ovarian steroid hormone levels are important in normal sexual function in women, as they are in other species.

Sexual desire in women is a matter of great controversy. Although sexual arousal and desire can be defined by subjective reports, only arousal has been defined objectively (as increased genital blood flow). There is not yet an objective measure of desire, thereby forcing it to be inferred from subjective self-report or intuitively observed behavior (e.g., flirtations). Desire appears to occur spontaneously in some women whereas in others it occurs in response to the right male(s) or females(s) making the right verbal and nonverbal gestures in the right contexts. As mentioned above, self-reported desire peaks during ovulation. This makes antecedent hormonal conditions—effects of estradiol, testosterone, and perhaps also progesterone, in the brain—likely motivational variables in its stimulation. Responsive desire, or the ability of the "right" stimuli to activate incentive motivational pathways in the brain and excite attention and behaviors that are indicative of desire, is also activated by steroid hormones. Desire is then expressed both as a spontaneous motivation and an attention toward competent sexual stimuli.[2,43] In both cases, the emergent conscious awareness of sexual desire activates movement from distal to proximal to interactive, like the approach and solicitations of rats and macaques. Humans thus learn a baffling array of appetitive responses that work differently in different cultures and contexts, or differently within a single culture at different epochs, and indeed

differently with different people. And cultures constrain women's responses, and indeed their own knowledge of their own sexuality, to appropriate times and places. The brain must balance these excitatory and inhibitory influences to achieve some kind of optimal level for pleasure. And it must do this with hormonal influences weighing it toward excitation, especially during ovulation, and experience directing attention and behavior toward individuals and stimuli previously associated with sexual reward.

The copulatory patterns of women are also fraught with problems of interpretation. Although more behaviorally stereotyped than appetitive responses, consummatory patterns of copulation in humans are nonetheless extremely variable, even in cultures where certain positions (e.g., missionary) are proscribed.[10,20,140] Some heterosexual positions, e.g., woman on top, can maximize her ability to get optimal external and internal CLS, possibly along with direct stimulation of the cervix, from the male.[141] Other positions may embellish other stimulus zones, and thus engage different motor patterns to maximize the stimulation achieved. And of course, some women are extremely sensitive to external CLS, and can only achieve orgasm in that way, whereas others achieve orgasms with blended internal and external CLS.[142–146] Interestingly, there is no human analogue to the lordosis reflex, although lordosis-like positions can be observed in women being mounted from behind. The arching of the back, however, is not a hormone-induced and/or facilitated spinal reflex; those do not exist in humans, a fact that continues to limit the human clinical application of the neuroendocrine work done on lordosis in animals. Being "receptive" to vaginal penetration in women involves a conscious decision to expose the vulva and open it to penetration. Experience with orgasm or other types of sexual pleasure and intimacy leads to expectancies which also constrain the sexual positions and patterns of both men and women.[145]

ANATOMY AND PHYSIOLOGY OF SEXUAL SENSORY SYSTEMS

The autonomic and peripheral nervous systems work together to send sensory information about genital and erogenous sexual arousal and stimulation to the spinal cord and brain, from which conscious feelings of desire and pleasure are derived (Figure 50.6). Genital (clitoral and cervical, but also involving sensitive regions of the labia and anus), and erogenous (nipples, lips) stimulation typically requires the engorgement of erectile tissues with blood (a parasympathetic activation). This engorgement increases the somatosensory surface area upon which stimulation can induce a response, essentially making the female more sensitive to tactile stimulation. Copulation typically involves more focused and localized genital stimulation that culminates in the buildup of a threshold amount of sympathetic arousal that brings about orgasm (or homologous responses in animals), and immediate sexual pleasure that activates inhibitory mechanisms related to euphoria (e.g., orgasm/reward), satiety and refractoriness.

Autonomic Control

The autonomic nervous system[147,148] consists of three divisions, sympathetic, parasympathetic, and enteric (the latter of which controls the gut). The role of the sympathetic system is to up- or down-regulate homeostatic and cardiovascular mechanisms to prepare the body for action (sometimes referred to as "the stress response" or the "fight or flight response"). Such action can be defined in terms of good stress ("eustress") or bad stress ("distress") depending on the nature of the event.[149] In either case, the sympathetic system initiates immediate pupil dilation (allowing for greater processing of the visual field), increased heart rate and blood pressure, dilation of the bronchioles of the lungs (to increase oxygenation of the blood), constriction of blood vessels, and inhibited digestion. It also is responsible for orgasm once sexual stimulation is underway. The sympathetic system activates the adrenal gland (located just above the kidney) to induce a massive release of adrenaline from the adrenal medulla (which activates and potentiates sympathetic outflow in most organs all at once, except the gonads). Adrenocorticotropic hormone (ACTH) released from the anterior pituitary stimulates the secretion of glucocorticoids such as corticosterone or cortisol from the adrenal cortex (which increases glucose concentrations in the blood, inhibits inflammation that might occur in response to injury, and potentiates arousal in the central nervous system and phenomena like place learning in the hippocampus of the brain's limbic system). The sympathetic nervous system extends from the spinal cord between the thoracic and lumbar divisions, and consists of short preganglionic nerves that contain the neurotransmitter Ach which excites postganglionic neurons, and long postganglionic nerves that contain norepinephrine (which inhibits prolonged muscle contractions). The ganglia collect incoming preganglionic fibers and distribute the postganglionic fibers to the organs in the abdominal and pelvic regions.

In contrast, the parasympathetic nervous system opposes the actions of the sympathetic system at each organ within each division. Its role is to calm the system down after stress, although it can be activated within each division on its own (e.g., as bright sunlight induces constriction of the pupil). The parasympathetic division is literally around ("para") the sympathetic division, and extends long fibers of the cranial nerves III, VII, IX, and

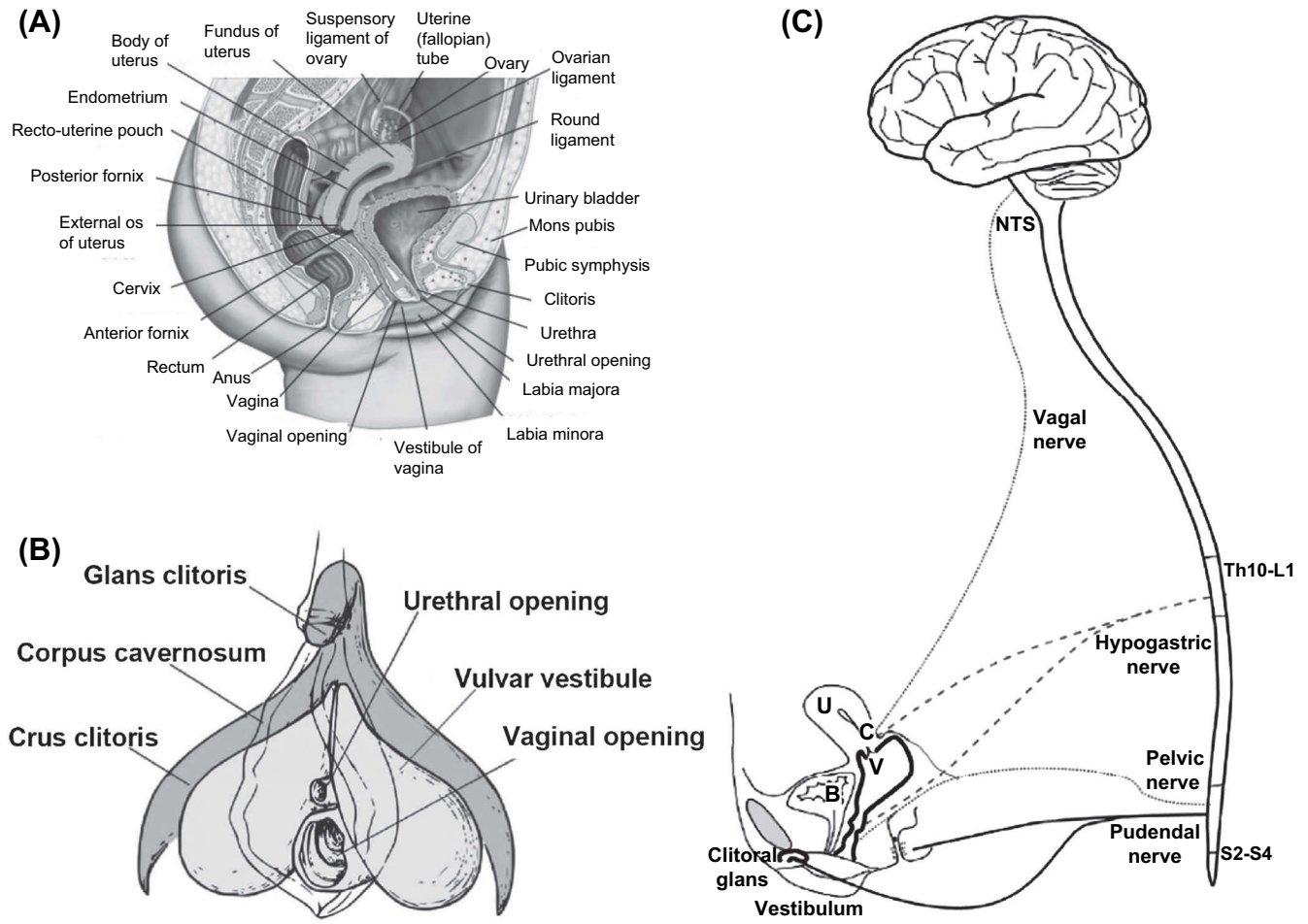

FIGURE 50.6 **Human female genital anatomy and neurophysiology.** (A) Cross-section of the human female genital and pelvic region. (B) The clitoral complex in relation to the urethra, vulva, and vagina. (C) Sensory nerve input to the spinal cord and brain from the genital and pelvic region, including pudendal, pelvic, hypogastric, and vagal nerve innervation. C, cervix; V, vagina; B, bladder; U, uterus.

X (occulomotor, facial, glossopharyngeal, and vagus) to innervate ganglia close to the organs. The postganglionic fibers that innervate the organs are short. Both pre- and postganglionic fibers contain Ach, which excites neurons and contracts smooth muscle. The pre-to-post relationship is more specific (e.g., 1:3) compared to the sympathetic division (which is greater than 1:10). Because the parasympathetic division causes dilation of blood vessels, it is critical for the stimulation of erection in labia, clitoris, and the vaginal epithelium, along with other erogenous erectile tissues (e.g., nipples and lips), and for draining the blood out of erectile tissues after orgasm.

Physiological sexual arousal can be defined as increased autonomic activation that prepares the body for sexual activity. In females this includes activation of the parasympathetic system that keeps blood in genital and erectile tissues, in particular the clitoris, labia, vaginal epithelium, nipples, and lips, and sympathetic blood flow from the heart to striated and smooth muscle that participate in sexual responses (Figure 50.7). Sexual arousal also includes a central component that increases neural "tone" or preparedness to respond to sexual incentives, and forms around an intricate interaction of hormone priming and noradrenergic activity in different regions of the brain. Both peripheral and central arousal may be detected as part of the perception of subjective sexual arousal, and both clearly lead to changes in responsiveness in genital tissues and control certain copulatory responses, such as the latency to orgasm (with shorter latencies indicating an increase in arousal). Both aspects are sensitized by estradiol and testosterone,[150] and are thus more likely to be experienced during the periovulatory period.

Peripheral autonomic blood flow is typically experienced far more readily than internal flow. Thus, women are less likely to be consciously aware of blood flow to the labia and clitoris relative to blood flow to the nipples. The reliance on vaginal arousal as a measure of sexual arousal may well account for the relative lack of concordance between physiological and subjective measures of sexual arousal in women relative to the concordance observed between physiological and subjective measures of sexual arousal in men.[151]

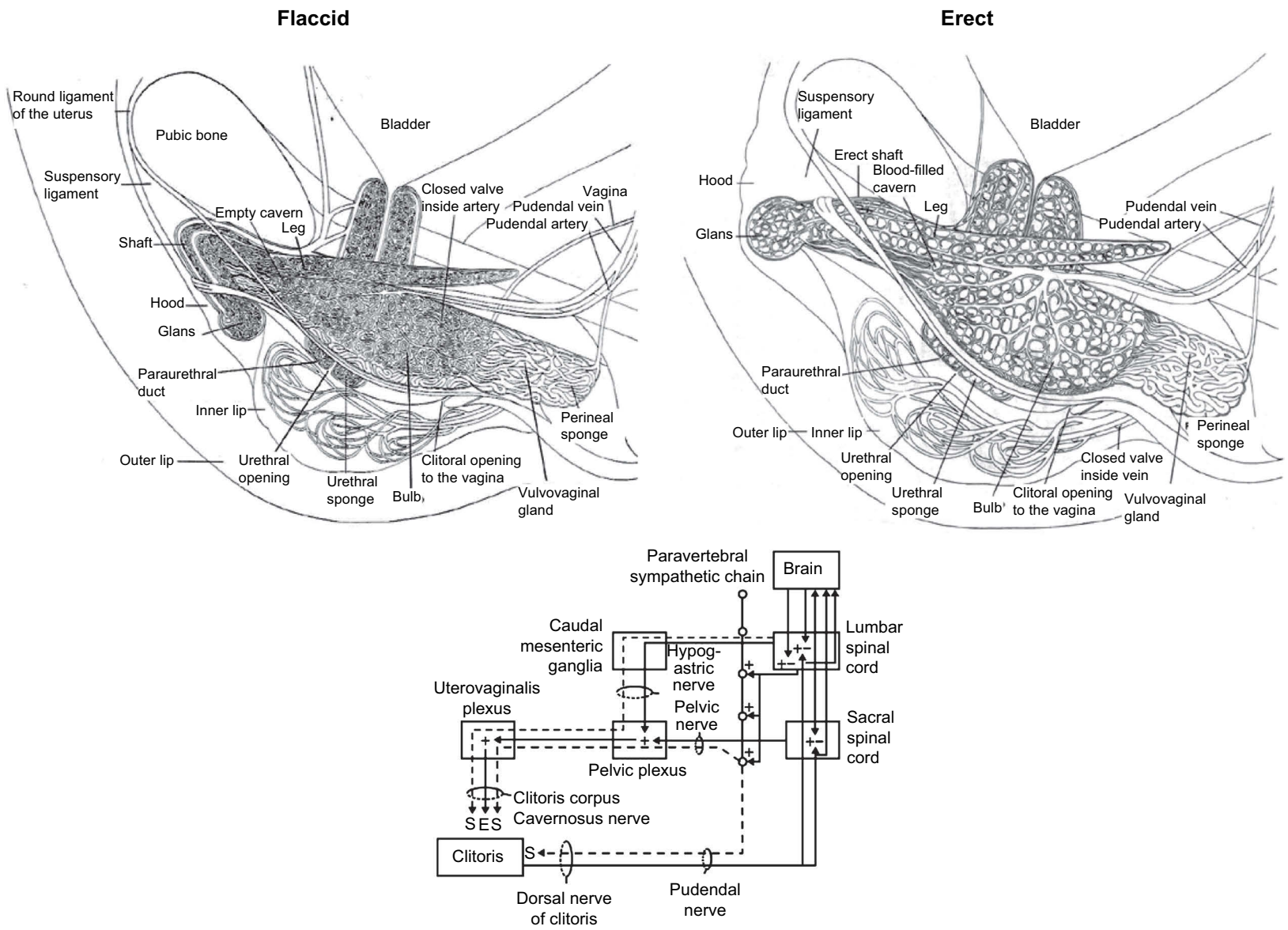

FIGURE 50.7 Top: The clitoral complex in its flaccid and erect states. Bottom: Wiring diagram of the sensory and autonomic pathways of the clitoral complex.

Genitosensory Stimulation

Both psychogenic and physical stimulation induce sexual arousal and desire that can lead to sexual behavior. Under normal conditions peripheral genital stimulation enhances excitability within sensory afferents that innervate spinal cord neural circuits. These spinal pathways contain the neural components for reflexive vasodilatation and orgasm and receive and send information to brain regions that perceive and modulate the signals that drive or inhibit sexual behavior. Although there are important differences in the brain and spinal pathways that mediate sexual function in males and females, largely related to sexual differentiation due to gonadal steroid hormone actions during fetal development (See Chapter 47), there is also substantial similarity between the CNS mechanisms mediating sexual desire, arousal and orgasm in males and females. In females, these include mechanisms involved in genital and psychological arousal, vasodilatation in the clitoris and vagina, smooth and skeletal muscle contractions, and rewarding sensations including orgasm.

Clitoral Stimulation

The clitoris is the main genitosensory organ of sexual pleasure in females,[130,132,134] and CLS influences vaginal muscle function.[152] It has been studied in rats with regard to its innervation and vasculature during sexual arousal[153–156] and its morphology in response to hormones.[157–159] However, its role in carrying sensory information during sexual activity in female rats has only recently been eludicated.[160–163] By engaging in lordosis, female rats not only receive vaginal intromission, but also CLS during mounts with pelvic thrusting. This interaction stimulates the female's flanks, rump, tailbase, perineum, and perivaginal surfaces which include the clitoris.[164] Intromission also stimulates the internal end of the clitoris in a region that overlaps in humans with the so-called "G-spot".[165–167]

In addition to CLS induced by male pelvic thrusting, direct CLS can be applied by an experimenter that mimics the distributed CLS a female rat might receive during copulation[160–163] (Figure 50.8, bottom left). Such stimulation on its own is rewarding in sexually naïve rats and stimulates preference for places and scents on partners associated with the reward state (see Section Consequences of Sexual Stimulation below). In contrast, CLS does not induce a preference in sexually experienced rats,[162] although it stimulates approach and solicitations of males behind a wire mesh screen.[161] Interestingly, the reward value of CLS is independent of steroid hormone priming, working as well in OVX rats as it does in OVX rats primed with estradiol alone or estradiol and progesterone.[162]

Clitoral afferents travel largely via the pudendal nerve (which branches into the dorsal penile nerve in males and dorsal clitoral nerve in females). This nerve provides the major sensory input from the external and internal clitoris and vagina, and provides the efferent (motor) innervations of the striated muscles of the pelvic floor and perineum.[170–172] Stimulation of the sensory branch of the pudendal nerve elicits vasodilation of the clitoris and vagina.[158,172–175] The increase in vaginal blood flow in response to sensory pudendal nerve stimulation was reduced by bilateral pelvic nerve cuts, suggesting that somatic afferents activate automatic spinal pathways to mediate the changes in vaginal blood flow that lead to vasocongestion.[174] The sensory field of the pudendal nerve is augmented by treatment with estradiol in OVX rats.[176]

Cervical Stimulation

The cervix is also stimulated during sexual interaction. Female rats, for example may receive VCS directly with each intromissive pelvic thrust from the male, whereas in humans the penis is generally not long enough to do this in most male-above positions, although it can occur in female-on-top positions.[177] Notably, however, orgasm in women also induces contractions of the cervix as part of what has been referred to as the "up-suck" reflex that aids sperm transport into the uterus.[178]

In rats small amounts of VCS potentiate lordosis whereas large amounts stimulate the termination of estrus. This stimulation-dependent excitation or inhibition depends on the hormonal status of the female and whether she can pace the copulatory contact with the male, as pacing increases the force with which the male intromits.[179–181] The augmentation of lordosis by small amounts of VCS is thought to be mediated at least in part, by the release of norepinephrine and possibly DA in the hypothalamus.[182,183] It may also be mediated by the release of OT in the spinal cord,[184] an effect that could also induce cervical dilation in preparation for sperm transport.

VCS can be applied by the experimenter using a smooth glass rod (Figure 50.8 bottom center) or plastic 1 cc syringe that approximates the width of the erect male rat penis. This stimulation can partially mimic the effects of intromissions by a male rat on reproductive physiology and behavior. This is somewhat surprising, however, because the probes provide pressure directly to the uterine cervix with mild distension pressure on the vaginal wall, whereas the erect rat penis is covered with keratinous spines,[185,186] which may more potently stimulate the vaginal wall even if the glans of the penis does not actually make contact with the cervix during intromission. Nevertheless, experimenter-administered VCS has many of the same effects on behavior and physiology as intromissions and is often used as a tool to simulate in a controlled manner the intromissions by the male. For example, experimenter-applied VCS increases heart rate[187] and elevates pain thresholds in both rats[188] and women.[189] VCS also

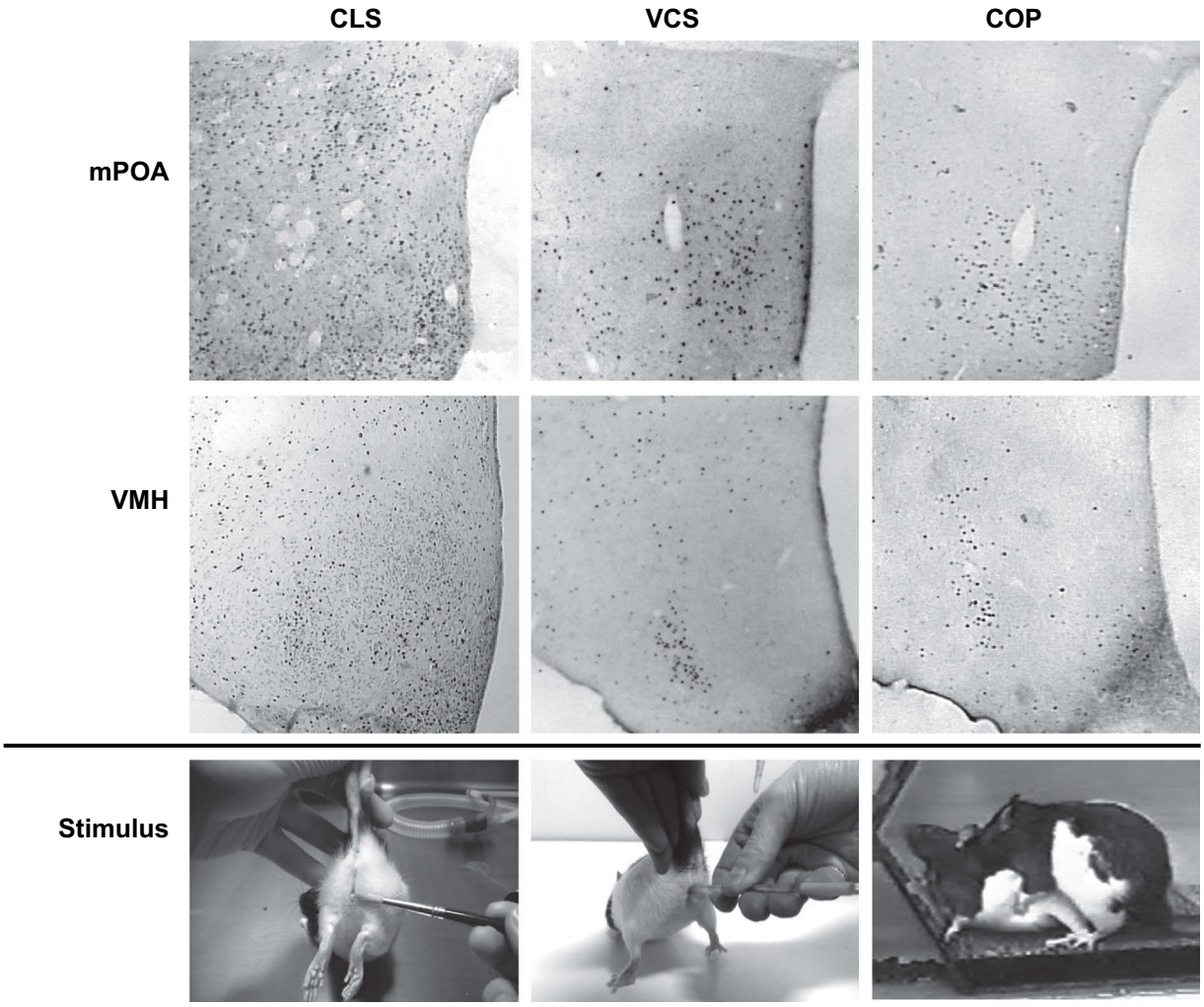

FIGURE 50.8 Fos induction in the mPOA (top) and VMH (middle) of OVX hormone-primed rats that received 50 distributed CLSs applied with a paintbrush,[160–163] 50 distributed VCSs applied with a lubricated glass rod,[75,168,169] or an hour's worth of paced copulation in bilevel chambers (bottom).

potentiates the ability of flank stimulation to induce lordosis in OVX rats not given hormone replacement,[190] and represents the only known stimulus, other than estradiol, to permit flank stimulation to induce lordosis.

Study of the relative contribution of intromissive, as compared to flank and perineal, stimulation provided by males suggested that nonintromissive stimulation is sufficient for mating-enhancement of lordosis, ear-wiggling and darting-and hopping in OVX/adrenalectomized (ADX) rats in a repetitive mating situation.[179] Therefore, VCS is not the proximate cause of mating enhancement in the repetitive mating situation, because, mounts without intromission were sufficient to enhance lordosis in OVX/ADX, estradiol-treated rats in the absence of progesterone. This makes logical sense; enhancement precedes the receipt of intromissions, because intromissions require that females display lordosis in response to mounts. Of course, the female does not show this posture until she is sexually receptive. In some experiments, rats have been OVX, and in others, they have also been ADX. Therefore, the interactions between adrenal ovarian hormones in the regulation of sexual responsiveness[182] and in the neuronal response to mating stimulation[191] must be considered. In some neuroanatomical areas, for example, OVX/ADX rats express the immediate early protein, Fos, in fewer cells in response to mounts without intromission than do OVX rats, but they express Fos in more cells in response to intromissions. This finding suggests that adrenal secretions may decrease sensitivity to low levels of mating stimulation.[191] Besides its influences on behavior, VCS influences luteinizing hormone (LH) release[192] and the twice daily surges of prolactin that then result in an extended period of diestrus[193] called pseudopregnancy or the progestational state.[194] More will be explained about the influences of VCS on estrus termination below. Cervical afferents travel largely via the pelvic nerve, and ablation of the pelvic nerve abolishes Fos induction in the brain by both VCS and copulation.[195]

Clitoral and Cervical Overlap

Both pelvic and hypogastric nerves convey sensory and noxious information from the internal reproductive organs, vagina and skin.[196] The cavernous nerve regulates both penile and clitoral erections. Stimulation of pelvic nerve afferents evokes an increase in vaginal blood flow, modeling genital arousal.[158] Vagal afferents from the uterus and cervix provide direct connections to the brainstem, and may sense orgasmic responses after spinal cord injury.[197] The distribution of the sensory afferents in the pelvis allows them to transmit considerable information relevant to mating, such as initiation and strength of sexual arousal, the location and movement of the penis inside the vagina, and orgasm or other pleasurable sensations derived from CLS. The clitoral glans contains specialized nerve endings that become very sensitive during erection and likely enhance sensation during intercourse, as is the case of the glans penis.[198,199]

Spinal Pathways

The sympathetic, parasympathetic, and somatic branches of the nervous system are coordinated and interconnected through important spinal pathways. This coordination allows sensory inputs to produce the appropriate sexual response, e.g., vasodilatation of erectile tissue, lubrication, increased sensitivity of erogenous zones, and muscle contractions during intromission and orgasm. In females the sympathetic spinal regions in lower thoracic-lumbar segments play a role in psychogenic arousal that occurs when distal cues (e.g., erotic visual cues in women; olfactory cues in rats) induce vaginal congestion.[200,201] However, genital responses occur only if the lumbosacral spinal cord that contains the pelvic and pudendal afferents and efferents is intact. Brainstem nuclei in the nucleus paragigantocellularis exert a serotonin-mediated tonic inhibition over the spinal pathways, and different regions of the brain (cortex, limbic system, mPOA) can be activated (directly or through visual or olfactory sexual stimulation) to initiate genital responses (reviewed in Ref. 196).

The pudendal nerve and pelvic nerve afferents course along the medial and lateral dorsal horn, respectively, of L5-S1 of the rat, with some afferent fibers penetrating into the dorsal gray commissure, and toward the parasympathetic preganglionic nucleus. In contrast, the hypogastric nerve afferents of the rat are relatively sparse and terminate in the superficial dorsal horn and medial gray of T13-L3.[171,195]

Fos Induction

Copulation, artificial VCS or CLS, urethral stimulation, or electrical stimulation of pudendal or pelvic nerves, all activate the immediate-early gene product Fos in neurons in similar spinal segments and subregions.[175,202] Activated spinal neurons are located in the superficial dorsal horn, the dorsal gray commissure of L5-S1 segments, overlapping with pelvic and pudendal sensory nerve innervations. Additional spinal neurons are activated in the intermediate gray and lateral gray dorsal to the parasympathetic preganglionic neurons. The distribution of activated spinal interneurons is similar in males and females. In parallel, electrophysiological studies identified similar spinal interneurons in the medial lumbosacral spinal gray matter after stimulation of the pudendal nerve and pelvic viscera.[203,204] However, VCS and CLS produce a different pattern of Fos activation in the rat brain (Figure 50.8, top). For example, distributed CLS that females find rewarding activates the medial nucleus of the mPOA,[160] whereas VCS activates a more medial region near the ventricles of the mPOA.[168,169] Paced copulation with a male (Figure 50.8, bottom right) activates both. In the VMH, CLS activates Fos throughout the dorsal and medial region, whereas VCS activates Fos exclusively in the ventrolateral region. Similarly, CLS activates Fos in limbic structures such as the posteroventral region of the medial amygdala (MEApv), whereas VCS activates Fos in the posteriordorsal region of the MEA.[18,160] In women, fMRI studies show that clitoral, vaginal, or cervical self-stimulation activates different regions of the sensory cortex.[205] Each of these are clustered in the medial paracentral lobe, a region that registers stimulation of the penis in the classic sensory homunculus of men.[206]

Tract Tracing

Neuroanatomical tract-tracing studies using neurotrophic viruses that are transported transneuronally through several synapses (for example, pseudorabies virus) have been used to map spinal and brain neurons that innervate the perineal muscles, clitoris, vagina, and uterus.[171,207–210] The majority of labeled neurons in the spinal cord are located in the dorsal gray commissure and in the vicinity sympathetic and parasympathetic preganglionic neurons, in the same regions as the Fos activated neurons. These neuroanatomical and electrophysiological studies reveal that genital afferents synapse on multiple interneurons in the spinal cord which then relay through a spinal pattern generator the preganglionic and postganglionic neurons and motoneurons to mediate, enhance, trigger, and maintain genital sexual responses. This complex spinal system allows coordination of sexual reflexes and sensorimotor modulation of these responses by different hypothalamic, midbrain, and brainstem regions (Figure 50.9).

Ultrastructural Changes in the Brain by Copulatory Stimulation

Sexual behavior itself influences neuronal morphology in rats[211] and hamsters.[94] After 1 h of mating in rats, there is a dramatic increase in expression of the cytoskeletal

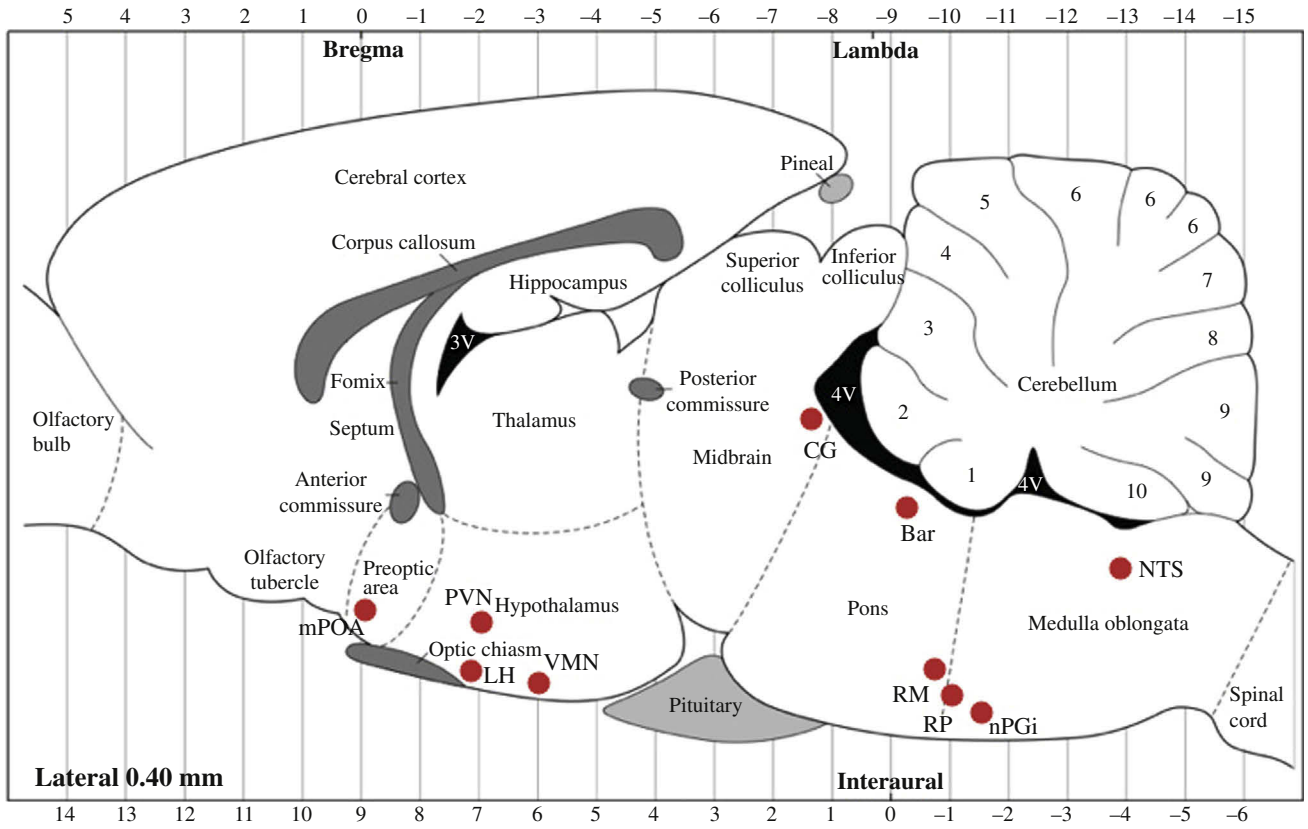

FIGURE 50.9 Dots depict areas of transneuronal staining in the brain 4 days after an injection of pseudorabies virus (a retrograde tracer) to the glans clitoris. *(Source: Adapted from Marson.[171,209])* mPOA, medial preoptic area; PVN, paraventricular nucleus; LH, lateral hypothalamus; VMN, ventromedial hypothalamus; CG, central gray; Bar, Barrington's nucleus; RM, medial Raphé; RP, posterior Raphé; nPGi, nucleus paragigantocellularis; NTS, nucleus of the solitary tract.

protein, Arc, in the ventrolateral VMH. Surprisingly, although the short-term effects were not assessed, this treatment also leads to a reduction 5 days later in secondary dendrites in this area. In hamsters, sexual experience increases the density of dendritic spines (membranous protuberences from the dendrite that typically receive a single synaptic input) in the prefrontal cortex (PFC), while decreasing it in the NAc.[94] Corresponding neurochemical changes have also been reported as a function of copulatory experience, including sensitization of D1 DA receptors[212] and Fos expression[213] in the NAc. It is clear that environmental stimulation, including that from mating, causes structural changes in neuroanatomical areas involved in sexual behavior. It is currently unclear how these changes relate to behavioral responses.

Other Erogenous Zones

Nipple and lip stimulation can be highly erotic during sexual arousal in both men and women, and can stimulate further sexual activity.[214,215] Nipple self-stimulation in women activates the genital sensory cortex (as well as the thoracic) region of the homuncular map in sexually experienced women.[205] The nipple/areola complex in women is innervated by the anterior cutaneous branches of the 1st to 6th intercostal nerves and laterally from the lateral cutaneous branches of the 4th intercostal nerve with additional innervation by cutaneous branches of the 3rd and 5th intercostal nerves (reviewed in Ref. 214). Innervation of the nipple appears to follow a pyramidal hierarchy, with most cutaneous sensory input in most women coming from the 4th lateral cutaneous branch, followed by the 3rd and 4th anterior branches.

Sensory innervation of the upper and lower lips comes from the maxillary and mandibular branches of the trigeminal (5th) cranial nerve, respectively.[216] Primary neurons send input to the trigeminal sensory nucleus, which is actually a sensory ganglion in the brainstem. Second order neurons project from there to the ventroposterolateral thalamus, and from there tertiary neurons carry input to the secondary somatosensory cortex (SII), in the lip region of the homunculus. The blood supply to the lips comes from the external carotid system,[217] which is aroused during parasympathetic activation of the lingual nerve. In the case of vasodilation in general for all genital and erogenous tissues, antihypertensive drugs

like nifedipine and propranolol can diminish erectile capability in both men and women.

In female rats, cutaneous stimulation of the flanks and perineum induces lordosis. Estradiol increases the area where cutaneous stimulation around the flanks and perineum induces lordosis, essentially sensitizing the somatosensory inputs to induce the reflex. This occurs by actions of estradiol in the periphery and within CNS modules that alter the propensity for cutaneous stimulation to induce lordosis.[32] Estradiol induces neurochemical and ultrastructural changes in the hypothalamic module (discussed below), notably in the VMH in concert with actions in other hypothalamic nuclei like the mPOA, that lead to an altered activation of neurons in the midbrain module of the central gray, which in turn activate neurons of the lateral vestibular nucleus in the brainstem module. This outflow activates the lateral vestibulospinal and reticulospinal tracts that synapse on motor neurons of the spinal module in the lower spinal cord (L5, L6, S1) to contract the lateral longissimus and transversospinalis muscles of the back, producing the characteristic arch of lordosis. However, such contractions are produced only when somatosensory afferents from the skin of the rump, tailbase, and perineum are activated by the male during anogenital investigation and more extensively during mounts with pelvic thrusting. Thus, although lordosis is essentially a spinal reflex, it is normally under tonic inhibition during periods of sexual nonreceptivity. The action of steroid hormones in the hypothalamic module is to constrain the lordosis reflex to an extended periovulatory period and disinhibit it in response to competent somatosensory stimulation of critical erogenous zones in the skin.

Primary Visual and Olfactory Senses

Sexual stimulation is often defined in terms of genital and erogenous somatosensory inputs (as those are explicitly and directly sexual), but largely separate from the main sensory systems that detect sexual incentives at a distance. In primates, and especially sighted humans, such stimuli are visual and auditory in nature. In other animals like rodents, they are olfactory in nature. Some stimuli are unconditionally arousing to human females (e.g., erotic pictures of attractive nudes or copulating couples[218,219]) or female rats (e.g., the smell of male rat fur[220]). Those unconditioned stimuli evoke attention and sexual approach behaviors like nose-pokes in rats. Treatments that disrupt olfactory input in rats, e.g., olfactory bulbectomy, zinc sulfate lesions of the olfactory epithelium, or olfactory occlusion with a polyethylene tube inserted into the nose, severely disrupt appetitive solicitations in females, but do not alter lordosis if the female is mounted.[221] Such treatments block copulation in male rats if they are sexually naïve, but not experienced.[28] Male olfactory cues alone activate Fos in the main olfactory (piriform) cortex, mPOA, VMH, and MEA of sexually experienced OVX rats primed with estradiol and progesterone.[221]

Erotic visual cues are used in studies of sexual arousal and desire in women (and men), and include still pictures or videos of nudes or different types of sexually explicit heterosexual or homosexual interaction. These are also used in brain imaging studies to correlate brain activation using fMRI or PET. Women experience cyclic fluctuations in sexual attention and arousability. For example, Mass et al.[60] reported pre-menopausal women's self-reported sexual desire and electromyographic responses of the facial zygomasticus major muscle (used for smiling and expressing joy) changed across the menstrual cycle during their exposure to pictures of naked men, with increases in smiling during the follicular phase and decreases during the luteal phase. Notably, these responses co-varied with increases and decreases in plasma progesterone, respectively. Similarly, event related cortical potentials (ERPs) recorded by scalp electrodes attached to the head that correspond to attention and stimulus processing for working memory increase in women following the presentation of sexually arousing pictures, but not pictures of babies or body care products, during the ovulatory phase.[59] The same pictures do not activate those ERP components during other phases of the menstrual cycle, or in women taking oral contraceptives.[222] Another study used fMRI to compare brain activation of premenopausal women in mid-luteal or menstrual phases of the cycle in response to erotic video clips relative to neutral video clips.[58,223] Increased activation by the erotic clips was observed in the anterior cingulate cortex (ACC), left insula, orbitofrontal (OFC), and parietal cortices, NAc, and hypothalamus, during the mid-luteal phase relative to the menstrual phase. These are virtually identical to the areas activated in men exposed to similar stimuli. The OFC is also activated by pictures of male faces, and the degree of activation is correlated positively with estradiol and progesterone levels in blood. The augmentation of the OFC response predicts the perceived attractiveness of the faces.[61] The ability of erotic visual stimuli to activate limbic and cortical structures is reduced after menopause, but can be restored to premenopausal levels following combined estradiol and testosterone treatment.[138] Thus, timing, context, and hormonal milieu, are extremely important variables to bear in mind when studying sexual arousal and responses to visual sexual stimuli in women.

HORMONAL PRIMING AND CONTROL

Steroid hormone synthesis in the ovaries is under the control of follicle stimulating hormone (FSH) and LH that are released from the anterior pituitary in response

to gonadotropin releasing hormone (GnRH) (see Chapter 28). FSH stimulates the growth of the ovarian follicle (and egg). When the follicle reaches a certain level of maturation it begins to secrete estradiol. LH causes the rupturing of the mature follicle to release the egg. The follicle becomes the corpora lutea ("yellow body") which then synthesizes and releases progesterone. The peak in estradiol secretion by the ovaries is followed by a small but critical rise in circulating androgens, notably testosterone, which is tightly linked in time to ovulation, and which, either alone in some species or in concert with the actions of preovulatory progesterone (from the follicle), activates appetitive approach and solicitation behaviors in many species. In humans, such behaviors are consonant with an increase in sexual desire. In most species, the expression of female sexual behavior is tightly regulated by ovarian hormones and occurs only during the periovulatory period.

Lordosis is perhaps the most-characterized and studied model of the hormonal regulation of behavior. In fact, the hormonal and neuroendocrine regulation of lordosis is similar in many species, including rats, mice, guinea pigs, hamsters, and gerbils. During the estrous cycle of these species, the secretion of estradiol followed by progesterone from the ovaries results in a period of sexual behavior that is tightly linked to ovulation.[65,224–226] Removal of the ovaries results in the loss of expression of female sexual behaviors.[65,227] High levels of sexual behavior in estrous cycling or OVX animals require estradiol priming followed by progesterone.[226,228] After behavioral estrus ends, sexual receptivity is not expressed until the next proestrous stage of the estrous cycle at which time estradiol secretion followed by progesterone once again induces sexual behavior. Although females of each of these species[229–232] may respond to estradiol alone, estradiol followed by progesterone is typically necessary for the expression of the full suite of female sexual behaviors closely resembling that seen in estrus-cycling animals.[14,65,225,227,233,234] The increase in both lordosis and appetitive sexual behaviors also occurs in OVX rats primed repeatedly with estradiol alone[84] and in ovary-intact, aged rats treated with testosterone.[235]

In some cases, after exposure to progesterone, rats,[236] hamsters,[231] guinea pigs,[237] and mice[238] become refractory to further stimulation of sexual behavior by either progesterone alone or, in some cases, to estradiol and progesterone. Although progesterone is believed to cause heat termination in guinea pigs, the role of progesterone in termination of sexual behavior during the estrous cycle of rats[239] is unclear. Based on work in guinea pigs to be discussed later, it has been suggested that progesterone desensitizes its response to itself, leading to termination of sexual receptivity and subsequent facilitation requires additional exposure to estradiol. Estradiol priming of behavioral response to progesterone generally takes about a day,[240,241] However, an intravenous injection of progesterone may facilitate the expression of lordosis within an hour of injection in estradiol-primed rats.[242–245] Interestingly, latencies as brief as 10 min for progesterone facilitation have been reported.[246]

Steroid Hormone Receptors

Although other hormones are involved, estradiol and progesterone have been the most extensively studied in the regulation of female sexual behavior in a variety of rodent species. This regulation involves a now "classic" steroid hormone action on specific intracellular receptors that act as transcription factors to induce gene expression (see Chapter 9). In turn, this sets up the excitatory and regulatory functions of the sexual brain, altering neurotransmitter synthesis, release, binding and reuptake, and altering synaptic connections in critical regions. Because of their role in the regulation of sexual behavior, understanding the regulation of these receptors is essential. A basic principle is the homologous and heterologous regulation of the receptors, each essential to ensure the critical timing of behavioral events with ovulatory events. This, in turn, sets up different neurochemical actions that time the onset, duration, and offset (inhibition) of the behavior.

A wide variety of steroid binding proteins has been described in the brain. These include the classic (so-called, nuclear) receptors that have been most extensively studied: ERα, ERβ, progesterone receptor A (PR-A; often referred to as progestin receptor A), progesterone receptor B (PR-B), and androgen receptor (AR). In addition to these classic receptors, other receptors, notably cell-surface receptors, have been described more recently that also mediate the effects of steroid hormones in the brain. Although these novel receptors are of great interest, much less is known about their role in the regulation of sexual behavior. As models of the regulation of female sexual behavior that involve the novel receptors develop, they must also be able to account for the data demonstrating the importance of the classic steroid hormone receptors as well.[247]

Classic ERs and Their Distribution in Brain

As described above, several subtypes of ERs exist, including the well-characterized classic (primarily) cell nuclear ERα and ERβ. Although relatively little is known about the neuroanatomical distribution of membrane-based ERs, a good deal is known about the distribution of ERα and ERβ. The following description of the brain localization of ERs focuses on the pattern of ERα and ERβ expression.

Four independent techniques have convergently revealed the distribution of ovarian steroid receptors

in the mammalian brain: in vitro binding, receptor autoradiography, immunohistochemistry, and in situ hybridization. These techniques measure the anatomical distribution of receptor binding activity, protein or mRNA expression, respectively, providing compelling evidence for regionally specific expression of these receptors. Table 50.1 summarizes the brain regions with the highest levels of ERs, based on these studies. Although most mapping studies have used rats, mice and guinea pigs, partial mapping of either ER protein or mRNA has been done in many other species, ranging from fish[248] and lizards[249] to musk shrews,[250] sheep,[251] human,[252,253] and nonhuman primates,[254,255] and the overall pattern is conserved.

The first studies delineating the anatomical distribution of estradiol binding used steroid receptor autoradiography with 3H-estrogens injected systemically. Although the initial observation of binding of an estrogen in the brain was made in cats,[256,257] in more comprehensive experiments,[258,259] regions of the hypothalamus and amygdala were especially rich in ERs. With the later discovery of distinct α and β subtypes of nuclear ER, autoradiographic studies were performed in transgenic animals with a "KO" of each of the subtypes of the ER.[260] These studies confirmed distinct neuroanatomical distributions for ERα and ERβ.

A great deal of the early work on the role of ERs in female sexual behavior and the biochemical characterization of them relied on in vitro 3H-estradiol binding in cell nuclear preparations of homogenates of particular brain areas.[261] In microdissection studies, the areas with the greatest density of ERs were in close agreement with autoradiographic studies showing ERs most abundant in the mPOA, VMH, and MEA.[262]

The early autoradiography and ligand binding studies on neural ERs relied on the binding of ^{3}H-estradiol to receptor proteins. Unfortunately, these techniques did not distinguish binding to each of the ER subtypes. However, the molecular cloning of the ERα and ERβ led to development of antibodies that could be used in immunocytochemical procedures that were able to distinguish the two subtypes. Immunocytochemical procedures were developed for both ERα[263–265] and ERβ.[266] Although immunohistochemistry provides the opportunity for superb subcellular localization, it has the disadvantage of not being able to distinguish between bound vs unbound receptors. This differs from autoradiography, which depends upon the receptor binding ligand. Thus, in addition to confirming the general pattern of receptor expression that had been revealed by receptor autoradiography, immunohistochemistry uncovered ERs in axons, dendrites, and terminals.[267,268] The presence of ER immunoreactivity in these nonnuclear sites complemented other evidence for nongenomic actions of estrogens, which will be discussed later.

The mRNA for ERα and ERβ has been mapped with in situ hybridization techniques,[269] and the neuroanatomical distribution of mRNA for the ERα and ERβ isoforms has been completed.[270] The results are in agreement with mapping studies based on receptor autoradiography in KO mice and subtype-selective immunocytochemistry. As a testament to the functional significance of the receptors in these regions, mating behavior produces a pattern of immediate early gene expression in the brain, a marker of neuronal activity, that corresponds to the neuroanatomical pattern of many locations of ER density.[168,271] Thus, ERs are well positioned to modulate the activity of the neural circuits that control mating behavior.

In summary, the neuroanatomical distribution of nuclear ERs is well documented, with the pattern of ERα expression coinciding well with brain regions known to

TABLE 50.1 The Dominant Pattern of ER Subtype Expression in Brain Regions Involved in Reproductive Behavior

	ER-Alpha	ER-Beta	Both
Amygdala	Amygdalohippocampal area		Medial nucleus
			Cortical nucleus
Septum	Subfornical organ		Bed nucleus stria terminalis
Hypothalamus	Median preoptic nucleus	Paraventricular nucleus	Medial preoptic area
	Anteroventral periventricular nucleus		
	Arcuate nucleus		
	Posterodorsal preoptic nucleus		
	Ventromedial nucleus		
Mesencephalon	Periaqueductal gray	Dorsal raphé	
	Locus coeruleus		
	A1, A2		

promote female sexual behavior. In a later section, we discuss the concept that in many areas with high levels of ERα expression, such as the VMH and POA, estradiol treatment increases the expression of the two forms of the progestin receptor (PR-A and PR-B).

Necessity of ERs for Hormonal Induction of Female Sexual Behavior

The notion that ERs are *essential* in order for estradiol to prime mice to become sexually receptive has been demonstrated by a wide variety of techniques, including: (1) injection of estrogen antagonists, which block the binding of estradiol nonselectively to ERs, or estrogen agonists that selectively activate either ERα or ERβ, (2) ER gene-disrupted mice (ER knockouts; ERKOs), in which the ERα or ERβ gene has been disrupted, and (3) RNAi silencing of ERα in specific brain regions.[272] The results of each approach are consistent with the conclusion that ERα is essential for the effects of estradiol on the expression of sexual receptivity, and that ERβ may have a modulatory role.

Effects of Estrogen Antagonists and Agonists

Early studies using estrogen antagonists to block the binding of estradiol to ERs were unequivocal in demonstrating the absolute necessity of binding to ERs in the mechanisms by which estradiol primes rats for female sexual behavior.[273,274] However, those antagonists were not specific for ER subtype. More recently, experiments using the ER subtype-specific agonists, propyl-pyrazole triol (PPT; ERα agonist) and diarylpropionitrile (DPN; ERβ agonist) indicate that ERα mediates the effects of estradiol on both appetitive and consummatory aspects of sexual behavior in female rats. Although the ERβ agonist is without effect when administered alone, it reduces the effects of the ERα agonist, suggesting that ERβ has a modulatory role in damping the effects of ERα activation.[275]

KOs and Knockdowns

KO strains of mice have been developed in which the gene for each form[276,277] or both forms[278] of ER is disrupted. Targeted disruption of the ERα gene (ERKO) completely eliminates hormonal induction of female sexual behavior.[279,280] In contrast, disruption of the ERβ gene (BERKO) was reported to be without effect in OVX, hormone-injected mice,[281] although it extended the period of behavioral estrus and enhanced receptivity.[282] Double KO mice with disruption of both, ERα and ERβ, exhibit decreased levels of sexual receptivity supporting the critical role of ERα in the sexual behavior of female mice.[281] Further support for involvement of ERα in female sexual behavior, and specifically its role in the VMH comes from the use of RNAi silencing of this gene in the VMH.[283] However, in a more recent study using the more complete ERβ-null mouse ($ER\beta_{ST}^{L-/L-}$),[284] complete elimination of ERβ resulted in a decrease in lordosis and attractivity in homozygous female mice.[285] Taken together, these experiments point to a critical involvement of both forms of the ER in the regulation of female sexual behavior in mice.

Regulation of ERs and ER Action

Because of the importance of ERs in the induction of female sexual behavior, it is essential to understand the ways in which levels of ERs in cells are regulated by estradiol and other compounds. There is some inconsistency among studies on regulation of ERα protein and mRNA levels by estradiol. However, estradiol has typically been reported to down-regulate ERα in most neuroanatomical areas.[286–291] Most studies find that estradiol also down-regulates ERβ in some neuroanatomical areas but is without effect in others.[289,292–294] Besides the homologous down-regulation of ERs by estradiol, ERs are down-regulated by progesterone[295–298] under some circumstances. Down-regulation in specific neurons that results from either hormone would be expected to decrease hormonal responsiveness in those neurons. The inconsistencies that exist in the literature with respect to the regulation of ERs by estradiol are to be expected, because there are numerous, important methodological differences between studies, such as doses of estradiol used, duration of exposure to hormone, time since OVX, etc. Furthermore, there is heterogeneity in the regulation of each form of ER, not just among neuroanatomical areas, but even among the neurons in a neuroanatomical area.[289] Nevertheless, such receptor down-regulation may help to explain why a substantial proportion of pre-menopausal women taking synthetic estradiol-containing oral contraceptives experience a blunting of their sexual desire, and an uncoupling of desire around the time that ovulation would normally have occurred.[299,300]

Pattern of Estradiol Exposure Sufficient to Induce Sexual Behavior

Acute Administration

The pattern of hormonal exposure is another critical variable in determining response to hormones. OVX rats need not be exposed to estradiol continuously during the priming period in order to express sexual behavior. Two pulses of a low dose of estradiol (e.g., 5 μg of estradiol benzoate, EB) spaced 24 h apart are more effective in inducing female sexual behavior after subsequent progesterone administration than a single higher dose of EB (e.g., 10 μg) or continuous exposure to estradiol from a silastic capsule implanted subcutaneously (sc) for several hours.[301–303] The behavioral effects of each pulse can

be blocked by either a protein synthesis inhibitor[304] or pentobarbital anesthesia,[305] suggesting that both protein synthesis and neuronal activity are required for each of the pulses of estradiol to be effective. The potential roles of classic and membrane receptors in these processes are discussed below.

Although continuous exposure to estradiol is not essential for the expression of sexual behavior, the continued presence of estradiol-bound ERs seems to be a requirement; administration of an estrogen antagonist that displaces receptor-bound estradiol inhibits sexual behavior, even when administered just prior to progesterone injection within a few hours of testing.[306] This finding suggests that the down-regulation of ERs by progesterone, which would then be expected to reduce ER-dependent estradiol action, may be part of the mechanism by which the period of estrus terminates.

Chronic Administration

Priming regimens in the literature vary, as do the strains of OVX rats used. It is clear, however, that a low dose of estradiol (e.g., 5 μg of EB) injected sc induces a moderate lordosis, but no appetitive approach or solicitation behaviors. The addition of progesterone (e.g., 100–500 μg sc) can increase lordosis further, and stimulate appetitive behaviors. However, continuous EB exposure through a silastic capsule implanted sc results in high and continuous levels of both appetitive sexual behavior and lordosis, as do frequent injections of EB, which sensitize both lordosis and appetitive sexual behaviors in sexually experienced female rats. Maintenance of serum estradiol concentrations above 15 pg/ml over a 5-week period with silastic capsules maintain normal body weight along with normal patterns of appetitive and consummatory sexual behaviors in female rats.[307] Serum concentrations below this cause increases in body weight and a dramatic suppression of appetitive responses with smaller decreases in lordosis.

The progressive elevation of appetitive and consummatory sexual behaviors by sc injections of estradiol is dependent on the dose and injection interval (Figure 50.10), such that as EB dose increases, behavioral sensitivity increases.[84,301,308–314] Notably, the sensitization is not blocked by ADX in OVX rats,[84] making it unlikely to be induced by facilitated release of adrenal progesterone. This finding is important because it further demonstrates that hormones can override inhibitory mechanisms that would otherwise act to inhibit sexual behavior as estrus terminates. A function of this increased sensitivity to the hormones, or decreased sensitivity to VCS, may be to ensure the female receives sufficient stimulation to maximize reproductive success.[75,84]

Estrogen sensitization is an important consideration in pharmacological studies that investigate compounds that might facilitate sexual behavior. Typically, OVX females are primed with a dose of EB that produces low-to-moderate amounts of appetitive sexual behaviors and lordosis. This is done to prevent ceiling effects that occur when animals are primed with EB and progesterone. However, animals are often tested repeatedly to reduce the number of animals used or because long-term treatment effects are of interest. The sensitization of sexual behaviors with chronic EB is confounding and makes data interpretation complex. This is particularly problematic in studies where the facilitative actions of a drug seem to disappear over time. EB dosing regimens have been characterized for OVX rats to achieve desired baseline rates of female sexual behavior across time[84] which will help overcome these problems. Stable baselines are vitally important in preclinical models of female sexual function. For example, 5 μg EB administered every 7 days, induces a stable baseline[84,315,316]; and when given in combination with flibanserin, a mixed 5-HT1A agonist/5HT2A antagonist, appetitive sexual behaviors were increased significantly by the drug following 3 weeks of treatment,[316] whereas the control group did not manifest any increase in behavior.

ER Co-regulators

Another level of regulation of response of a cell to a hormone lies in steroid receptor coregulators; intracellular proteins that allow for efficient regulation of the transcription of steroid receptors (see Chapter 9).[317] These proteins bridge the receptor and the general transcriptional machinery and modify promoter regions of the receptor by a variety of mechanisms. Although there are over 300 steroid receptor coregulators,[318] and we have much to learn about how they modulate the action of ERs, perhaps fine-tuning responses to steroid hormones, we do understand the role of three coactivators in regulation of female sexual behavior. Work using intracerebroventricular infusion of antisense oligonucleotides directed against the mRNA for particular steroid receptor coactivators in rats suggests that steroid receptor coactivator-1 (SRC-1) and cAMP response element binding protein (CBP) act together to modulate the induction of sexual receptivity by estradiol,[319] as well as the induction of progestin receptors[319] and progesterone-facilitated sexual behavior in female rats.[320] Likewise, SRC-1 and SRC-2 play an important role in the cellular action of estradiol in the induction of female sexual behavior in rats and mice.[321]

In order for coactivators to influence the activity of steroid receptors, they must be coexpressed in the same neurons as the receptors. In fact, SRC-1 and SRC-2 are expressed in most cells expressing ovarian steroid hormone receptors in the VMH, mPOA, and arcuate nucleus (ArcN) of female rats and mice.[317] Although

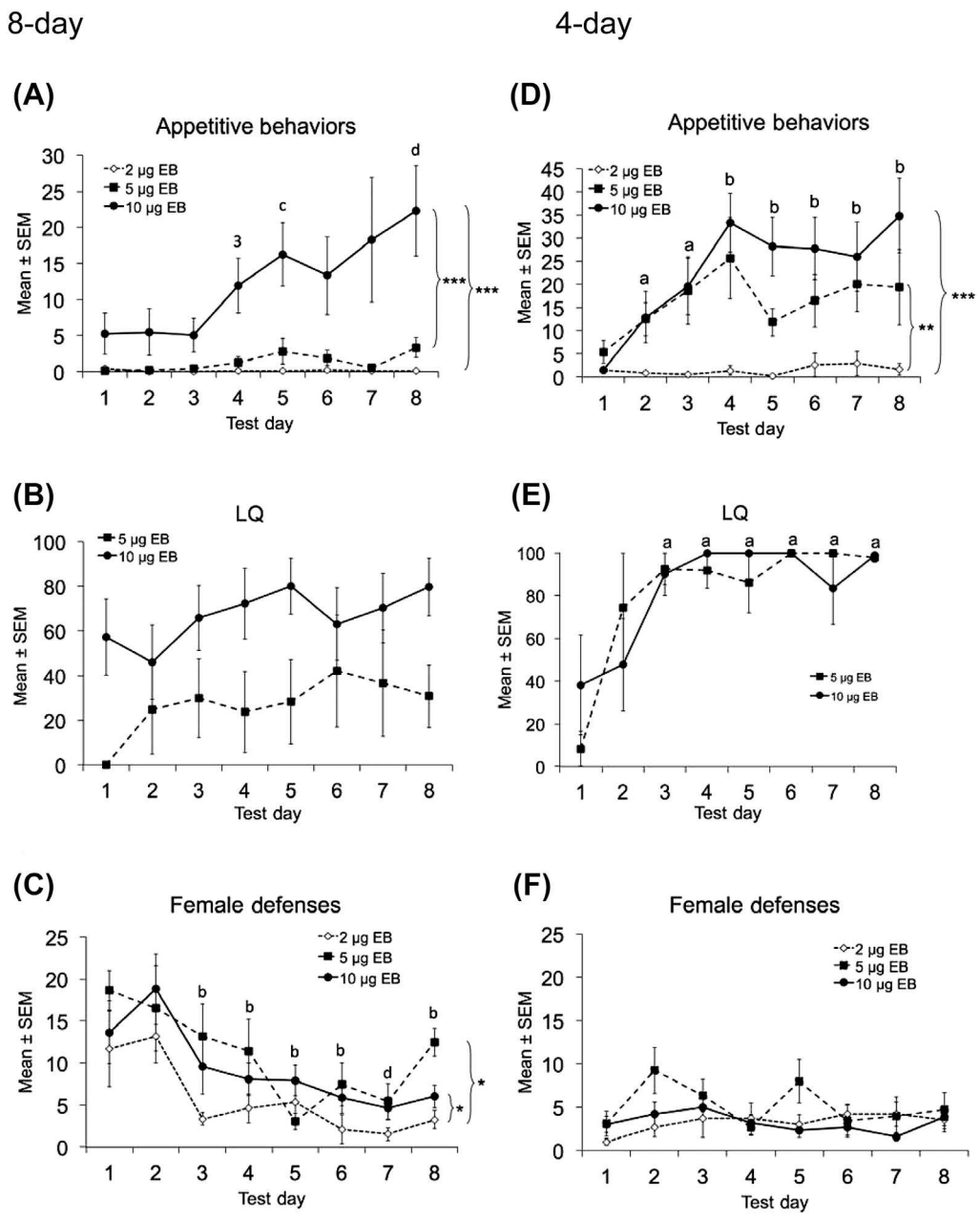

FIGURE 50.10 Sexual behaviors of OVX Long-Evans females treated with varying doses of estradiol benzoate (EB) at 8-day (left) or 4-day (right) intervals. (A) Appetitive sexual behaviors increased in females treated with 10 µg EB, but not 2 µg or 5 µg when treated at 8-day intervals. (B) Lordosis quotient (LQ) did not sensitize when treated with EB at 8-day intervals. (C) Collapsed across EB treatment group, females were less defensive toward males as of Test 3. (D) When treated at 4-day intervals sexually appetitive behaviors sensitized in females treated with 10 µg EB. (E) LQ sensitized in females treated with 5 and 10 µg EB when treated at 4-day intervals. (F) Defensive behaviors were unaffected when treated with EB at 4-day intervals. [a]Different from Test 1; [b]Different from Tests 1 and 2; [c]Different from Tests 1, 2, and 3. [d]Different from Tests 1–4. Numerical superscripts are used to indicate differences from specified test day. Brackets represent main effect of EB Group. $*p<0.05$, $**p<0.01$, $***p<0.001$. *Source: Reprinted from Jones et al.,[84] with permission of Elsevier.*

in vitro studies suggest that the relative expression levels of the coactivators and corepressors determine cell-specific, appropriate and graded responses to steroid hormones,[315] there has to date been no work on this subject in the brain and on behavior. It is however likely that this represents another level of fine-tuning of the hormonal regulation of female sexual behavior.

Is Binding of ERs to Estrogen Response Elements Essential?

Not all ER-mediated responses require the prototypical estrogen response elements on specific genes. A gene knock-in mouse model with a mutated ERα that does not bind to estrogen response elements (EREs)[322] expresses

negative, but not positive, feedback to estradiol on gonadotropin secretion, suggesting that negative feedback does not require binding of ERα to an ERE. While expression of masculine sexual behavior in males was shown to require binding to EREs,[323] results on female sexual behavior have not been reported. Nevertheless, this work suggests that some effects of estradiol that are relevant to female sexual behavior do not require binding the nuclear ER to an ERE, opening up the possibility that estradiol induces these effects through membrane ERs.

Role of Membrane ERs

Recent work has made clear that, while classic ERs are principal players in regulation of female sexual behavior by estradiol, membrane mechanisms are also involved. Although much less is known about membrane ERs than about the role of classic, so-called, nuclear ERs, there is now a good deal of evidence for the involvement of membrane mechanisms of action for the effects of estradiol on female sexual behavior. A variety of putative membrane ERs has been described,[324] including the classic ERα and ERβ, which can be translocated to plasma membranes,[325] ER-X, STX (a tamoxifen analogue that is estrogenic)-activated membrane ER, and GPR30 (G protein coupled estrogen receptor; GPER).

Although still in an early stage of research, the data argue strongly for the involvement of membrane ERs in the regulation of female sexual behavior by estradiol. For example, a biotinylated form of estradiol, which is impermeable to the cell membrane, interacts with metabotropic glutamate receptors in the mPOA, resulting in an increase in lordosis.[326] Specifically, membrane ERα[326] and/or the STX-activated membrane ER[327] is believed to interact with the metabotropic glutamate receptor 1a in the ArcN, resulting in the internalization of mu-opioid receptors in the mPOA and consequently an increase in lordosis via projections to the VMH. It should be noted, however, that while the STX compound facilitates the expression of lordosis in OVX rats, it only does so in rats administered a subthreshold dose of estradiol.[327] Therefore, the STX-activated mER participates in the process of estradiol priming of sexual behavior, but it is not sufficient to substitute for estradiol. As discussed earlier, administering estradiol in discrete pulses allows much lower doses of estradiol to be used to induce sexual behavior. However, little is known about the cellular basis for the enhanced response to pulsed exposure as opposed to bolus injection of estradiol. In some experiments, estradiol conjugated to bovine serum albumin (another form of estradiol which is impermeable to cell membranes), has been used.[328] The fact that this conjugated estrogen can substitute for either the first or later pulse of estradiol indicates that membrane ERs are capable of completing at least part of the priming for sexual behavior. Compounds that activate either protein kinase A (PKA) or PKC can also substitute for the conjugated estrogen,[328] suggesting involvement of these two intracellular signaling pathways in the priming action of estradiol on female sexual behavior. It should be noted that experiments using protein conjugates of steroid hormones must be interpreted very cautiously because of the possibility that the protein could be cleaved from the steroid molecule[329] and because the position of the protein on the steroid can have unexpected effects on function.[330] Nevertheless, the data argue for the ability of membrane ER mechanisms to substitute for interaction of estradiol with classic ERs.

With the exception of the work of Kow and others,[328] mechanistic studies have typically focused on the role of *either* nuclear ERs acting as transcriptional regulators or on the role of membrane receptors in regulation of sexual behavior. However in vitro, ERα and ERβ each may be processed to become associated with membranes, and are capable of signaling through the mitogen activated protein (MAP) kinase pathway.[331–333] This finding indicates that caution must be exercised in interpreting experiments which used hormone antagonists, antisense oligonucleotides or targeted gene disruption to test the involvement of ERs acting as transcription factors. If the ER genes that direct synthesis of the classic ERs also direct the synthesis of membrane receptors in the brain, then the manipulations that target the classic ER could disrupt membrane receptors as well as classic nuclear receptors.

As mentioned earlier, ERα immunoreactivity[267,334] has been observed in extranuclear locations within the guinea pig hypothalamus, including axon terminals and distal dendrites[267] (Figure 50.11). In some cases, this has been observed associated with synaptic densities and plasma membranes, which is consistent with the idea that classic ERs can be directed to membrane sites.

To summarize this section, in addition to actions of estradiol on classic ERs, estradiol may signal through a variety of membrane receptors to either substitute or perhaps, augment the behavioral effects of estradiol acting on classic ERs. There is still a great deal to be learned about the interplay of these various receptors in the fine-tuning of the hormonal induction of female sexual behaviors.

Classic PRs and Their Distribution in Brain

As with ERs, evidence from receptor autoradiography, immunohistochemistry, and in situ hybridization presents a coherent picture of the pattern of expression of nuclear PRs, although information about membrane receptors is still being gathered. Early receptor autoradiography experiments first revealed nuclear progesterone binding activity in the rodent brain.[335,336] Although progesterone binding was found in regions with an

abundance of ERs, such as the hypothalamus and amygdala, a few brain regions expressed PR in the absence of ER, suggesting separate functions. Double-label immunohistochemistry studies have shown that ERα and PR are often co-localized at the cellular level in brain regions known to control female sexual behavior.[289,337] The mapping of mRNA of the PR has been conducted using in situ hybridization techniques in rats, rabbits, lizards and fish.[338–341] Although there may be some species differences in regulation, the overall pattern of nuclear PR expression is conserved in regions associated with reproductive behavior, including the VMH, mPOA, median preoptic nucleus, anterior hypothalamic area, medial nucleus of the amygdala, anteroventral periventricular nucleus, lateral habenula, ArcN, and periaqueductal gray.[342,343] It should be noted many of the PR-immunoreactive cells in the VMH are located outside the Nissl-defined nucleus,[337] and these cells have been implicated in the facilitation of sexual behavior by progesterone, at least in guinea pigs.[344]

Although there is a great deal of overlap between neuroanatomical sites containing estradiol-induced PRs and those that contain ERs, immunocytochemical experiments demonstrated conclusively that estradiol-induced PRs are co-expressed in ER-ir cells in brain regions involved in regulation of female sexual behavior.[289,345] Virtually all cells expressing estradiol-induced PRs also express ERα (Figure 50.12).

The PR is synthesized from alternative, estrogen-inducible promoters on the PR gene, resulting in two isoforms of the receptor (PR-A and PR-B) with somewhat

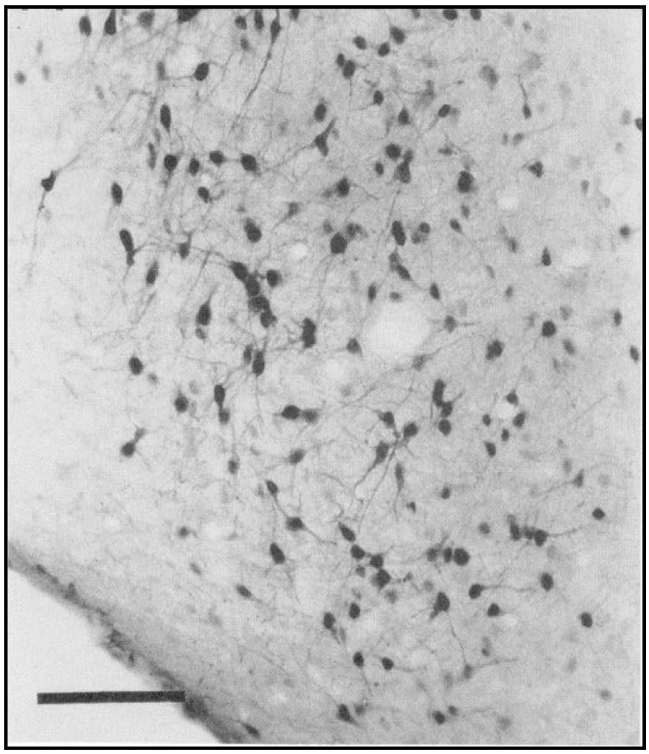

FIGURE 50.11 ER-immunoreactivity in the VMH of an OVX guinea pig, visualized by the silver-intensified, diaminobenzidine-peroxidase technique in vibratome-cut sections. Magnification bars = 100 μm. *Source: Reprinted from Blaustein et al.,[267] with permission of the Endocrine Society.*

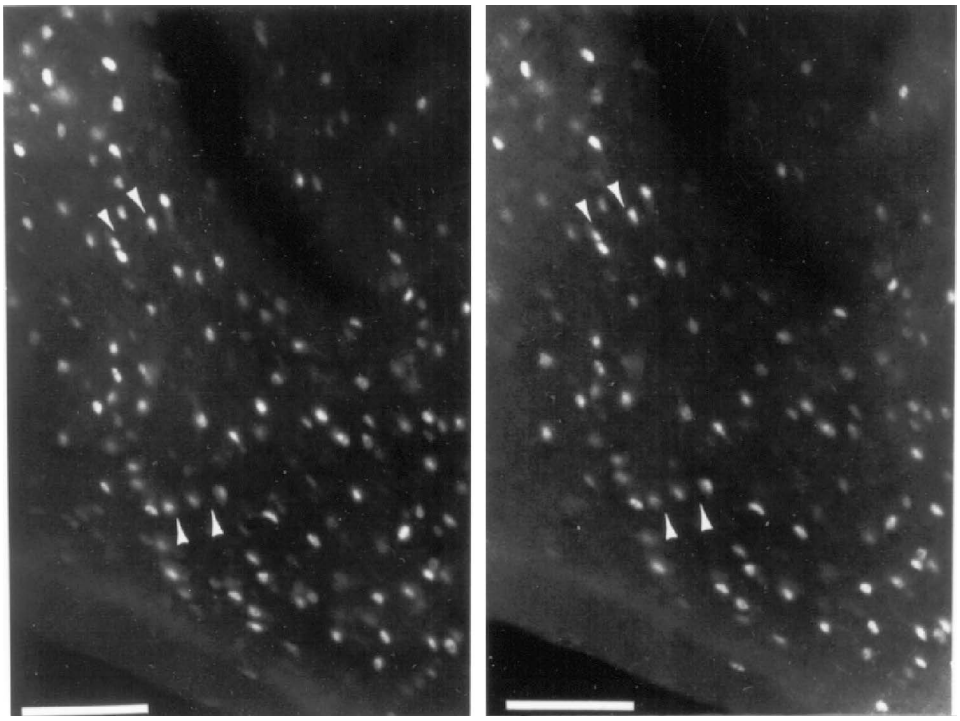

FIGURE 50.12 Photomicrographs of PR-immunoreactivity (right panel) and ERα-immunoreactivity (left panel) coexpression in the VMH of an estradiol-primed, OVX guinea pig showing that virtually all estradiol-induced PR cells also coexpress ERα-immunoreactivity. Arrowheads point to cells containing both estradiol-induced PR-immunoreactivity and ERα-immunoreactivity. *Source: Reprinted from Blaustein and Turcotte,[345] with permission of Karger.*

different transcriptional activities.[346,347] Although our understanding of the differential distributions of these PR subtypes remains incomplete, the two isoforms are expressed in the rat brain,[348] and the ratio of the two isoforms varies under different hormonal[349–352] and behavioral[353] conditions. Experiments using antisense oligonucleotides directed at the mRNA for each or both of the isoforms demonstrate differential regulation of particular genes by each isoform.[354]

Necessity of PRs for Progesterone Regulation of Female Sexual Behavior

The characterization of neural PRs suggested the hypothesis that PRs are essential for the facilitation of sexual behavior by progesterone.[355] This hypothesis predicted that sensitivity to progesterone is determined by the concentration of unoccupied PRs available in neurons involved in progesterone-facilitated sexual behavior, and response is dependent on an adequate concentration of activated PRs in those cells. An increased concentration of PRs (e.g., after estradiol priming) would be expected to increase the sensitivity of the neural substrate for progesterone, presumably by increasing the concentration of receptors that become activated in response to progesterone treatment. Likewise, a decreased concentration of unoccupied PRs would be expected to result in decreased sensitivity to progesterone. This PR hypothesis[356] was corroborated in later work on the regulation of PRs that will be discussed. A central mechanism involving neuroprogestin activation of PR, or ligand-independent activation of PR, has not been determined.

Upregulation of PRs

As discussed, estradiol increases the concentration of PRs in the hypothalamus, mPOA, and a number of other brain regions (Figure 50.13). Coincident with the increase in the hypothalamus, behavioral responsiveness to progesterone increases. The increased concentration of PRs and behavioral responsiveness to progesterone are both transient.[358,359] In gonadally intact, estrus-cycling rats, the concentration of unoccupied PRs in the hypothalamus increases during proestrus in response to estradiol.[244] Collectively, these experiments suggested that PRs are a critical aspect of the cellular mechanism by which progesterone facilitates sexual behavior.

The duration of sexual receptivity for each species is tightly regulated, lasting about 8 h in guinea pigs[360] and about 14 h in rats.[361] The timing of the *duration* of sexual receptivity is referable at least in part to the regulation of activated PRs in particular neurons.[356] The presence of activated PRs in particular neurons presumably leads to the expression of neuropeptides and neurotransmitters described below, but the presence of the activated

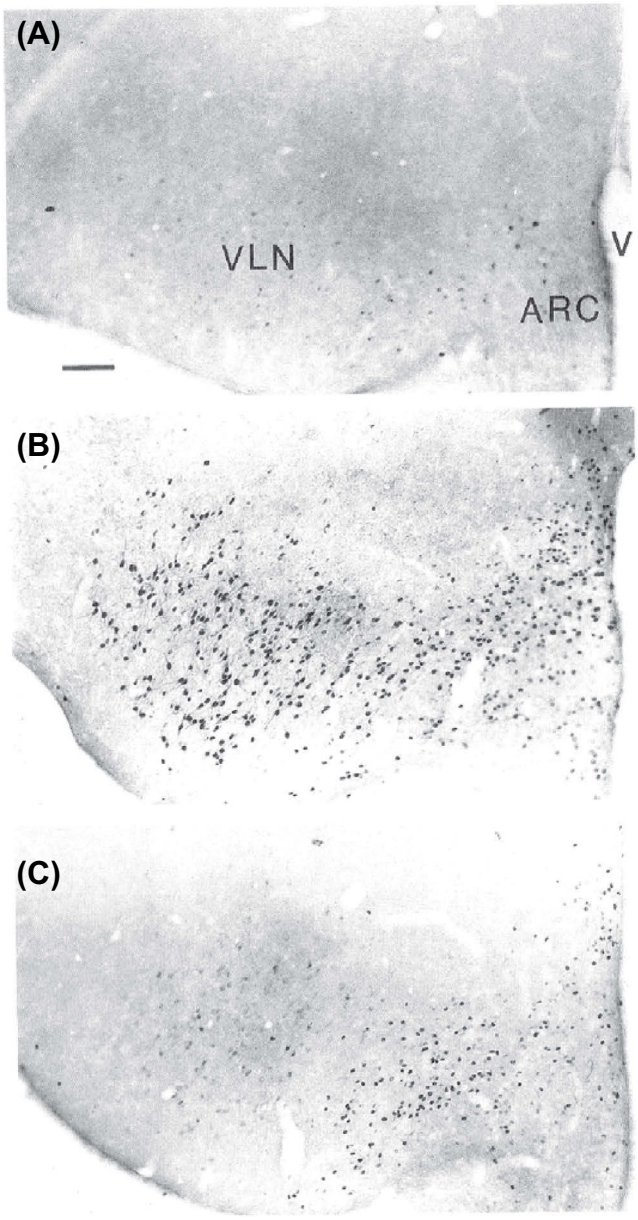

FIGURE 50.13 PR-immunoreactivity in the rostral aspect of the ventrolateral nucleus of the hypothalamus (VLN) and arcuate nucleus (ARC) of OVX guinea pigs injected with: (A) oil (0 h) and oil (42 h), perfused 24 h later, (B) estradiol benzoate (0 h) and oil vehicle (42 h), perfused 24 h later, or (C) estradiol benzoate (0 h) and progesterone, perfused 24 h later. Magnification bar = 100 μm. *Source: Reprinted from Blaustein and Turcotte,[357] with permission of Wiley.*

PR may act to gate the transcriptional activity of the relevant downstream genes. Progesterone injected in estrogen-primed, OVX guinea pigs and rats[244,362,363] or the preovulatory progesterone secreted during the estrous cycle[363] binds to and activates neural PRs. The presence of activated PRs in a pooled sample of the hypothalamus-preoptic area after progesterone injection correlates well with the ability of female rats to display lordosis.[362] Manipulations which prolong the period that

hypothalamic PRs remain occupied extend the duration of that period in female rats.[364,365] This temporal agreement between activated/occupied PRs and expression of lordosis suggests that it is maintained by elevated levels of occupied PRs, and that termination of lordosis is due at least in part to loss of these receptors.

Besides these correlational studies, a variety of techniques—injection of progestin antagonists, antisense oligonucleotides to PR mRNA and PR knockout (PRKO) strains of mice—has been used to demonstrate that PRs are *essential* for the facilitation of lordosis by progesterone. Systemic injection[366,367] or intracranial application[368] of a progestin antagonist inhibits the facilitation of lordosis by progesterone in rats and guinea pigs. However, because most antagonists are not completely specific, other techniques have been used to test the necessity of PRs for progesterone function in sexual behavior. Infusion of antisense oligonucleotides to PR mRNA, which inhibits PR synthesis, into the cerebral ventricles[369] or VMH[370,371] blocks facilitation of both appetitive sexual behaviors and lordosis by progesterone. Similarly a transgenic mouse strain with a targeted disruption of the PR gene (PRKO)[372] are completely unresponsive to progesterone for the facilitation of sexual behavior.[373] Using PR isoform-specific KO strains of mice to determine the relative contribution of each PR isoform to progesterone-facilitated female sexual behavior, Mani et al.[374] observed that progesterone-facilitated lordosis was completely eliminated in the PR-A null mutant mouse, and PR-B null mutant mice showed a trend of suppression of progesterone-facilitated sexual behavior. Although the specific function of PR-B is unclear, the data collectively suggest that PR-A is essential for progesterone-facilitated lordosis, and both isoforms are required for optimal facilitation by progesterone.

The question of which ER is involved in up-regulation of PRs has received some attention. The ERα selective agonist, PPT, induces PR mRNA, at least in the VMH and ArcN.[375] Although PR-ir induction in the brain by estradiol is dramatically reduced in ERαKO mice, KO of ERβ does not fully eliminate PR-ir induction.[281,376] Furthermore, genetic downregulation of ERβ (albeit in the incomplete ERβb KO mouse) was without effect on PR-immunoreactivity in the VMH. In subsequent work, in which it was confirmed that the ERα agonist, PPT, induces PR-ir in the VMH, it was also determined that the ERβ agonist, DPN does not. However, the sequential injection of an ERβ agonist after an ERα agonist induces PR immunoreactivity in more cells in the VMH than the ERα agonist alone.[377] Based on experiments using BSA-conjugates of estradiol, it has been suggested that membrane ERs are also involved in the induction of PRs in the VMH.[378] Collectively, these experiments point to a critical role for ERα, a possible role for a membrane ER, and a minor role for ERβ in the induction of PR immunoreactivity, at least in the VMH.

Membrane PRs and Nonclassic Mechanisms of P Action

The mechanism of action of progesterone is not as simple as activation of just PR-A and PR-B. Although the classic mechanism of hormone action plays an important role in the regulation of sexual behavior, membrane mechanisms are also involved.[379] Progesterone can also influence electrophysiology and facilitate sexual behavior within seconds and minutes, respectively. Some of these effects may be referable to activation of cell surface PRs, ion channels and cytoplasmic second messenger signaling cascades, and are independent of gene transcription.[380] Recently, membrane proteins unrelated to classic PRs have been characterized. The presence of membrane PRs (mPRs) and progesterone receptor membrane component 1 (PGRMC1) and PGRMC2 in the brain[342,381–384] provides a possible mechanism by which progesterone could have rapid effects on behavior and neurophysiology. mPRs are G-protein coupled receptor members of the seven trans-membrane adiponectin Q receptor family, and come in at least three subtypes.[385] In addition, PGRMC1 (also called 25Dx) is regulated by estradiol and progesterone in the VMH of female rats.[343,381] Although the direction of the regulation is not consistent between the two reports,[343,381] the hormonal treatments and methods used were quite different, which may explain the discrepancy.

PGRMC1, PGRMC2 and classic PR mRNAs are expressed at high levels and have a good deal of overlap in the mPOA and other hypothalamic nuclei and their projection sites.[342] Under some conditions, progesterone treatment results in an increase in PGRMC1 mRNA levels in the VMH and preoptic area.[343] Likewise, mPRα and mPRβ are present within the hypothalamus and preoptic areas, among other areas, and estradiol increases the expression of mPRβ[384] and the estrous cycle influences the expression of mPRα and mPRβ expression in some brain areas.[383] Although the functional role of each of these putative membrane receptors in hormonal regulation of sexual behavior remains to be determined, their presence in the brain suggests additional mechanisms by which progesterone could rapidly influence sexual behavior and possible interactions of mPRs and classic PRs within the same neurons.

Cross-Talk between Neurotransmitters and Steroid Hormone Receptors

Neurotransmitters Influence Concentrations of ERs and PRs

One of the most interesting aspects of the regulation of female sexual behavior is the interplay between external factors and the internal hormonal *milieu*. Because of the critical role of steroid hormone receptors in the

mechanisms of action of steroid hormones on female sexual behavior, studies of integration between afferent information and steroid-hormone sensitive systems have focused on the regulation of these receptors. The finding that catecholaminergic activity influences the concentrations of neural sex steroid receptors in rat and guinea pig brain[386] suggested that stimuli from the environment might regulate the concentration of steroid receptors in neurons involved in female sexual behavior, and consequently, behavioral response to hormones.

Drugs which either inhibit norepinephrine synthesis (dopamine-β-hydroxylase (DBH) inhibitors) or which block noradrenergic receptors (e.g., α-adrenergic antagonists) typically decrease the concentration of ERs in some neural areas[387,388] and/or inhibit induction of hypothalamic PRs by estradiol,[389–391] and α-adrenergic agonists reverse this suppression. Noradrenergic antagonists also decrease female sexual behavior in guinea pigs.[392] This, together with the finding that injection of an α_1-noradrenergic antagonist, which decreases ER concentrations in the hypothalamus, also decreases female sexual behavior,[393] suggests behavioral relevance of the neurotransmitter regulation of ERs. Finally, under some conditions, stimulation of DA receptors increases the concentration of ERs in the brain.[394,395]

A possible anatomical substrate for the integration between catecholaminergic neurons and steroid hormone-responsive neurons can be found in the catecholaminergic innervation of some ER-containing neurons,[396,397] and immunoreactivity for tyrosine hydroxylase and DBH, the enzyme that converts DA into norepinephrine (NE), varicosities are sometimes found closely associated with PR- or ER-immunoreactive neurons in the mPOA and hypothalamus.[398–400] The fact that those ER-immunoreactive cells with closely associated DBH-immunoreactive varicosities stain more darkly for ERs than other ER-immunoreactive neurons lacking this association suggests that noradrenergic input regulates the level of ERs in a population of these ER-immunoreactive cells,[400] and consequently, behavioral responsiveness to estradiol. It should be noted that neurotransmitter regulation of steroid receptor levels is not limited to the catecholamines. For example, muscarinic agonists and antagonists regulate the levels of neural ERs.[401]

Defined anatomical connections may influence steroid receptor levels and therefore, presumably, sensitivity to steroid hormones for their influence on sexual behavior. For example, anterior roof deafferentation using knife-cuts in female rats increases the behavioral response to estradiol, presumably referable to the resulting increase in the concentration of ERs in the mediobasal hypothalamus.[402] Conversely, olfactory bulb removal results in an increase in the concentration of ERs in the MEA in female rats, presumably related to the mechanism by which olfactory bulbectomy increases sexual behavioral response to estradiol.[403]

Input from the social environment also regulates steroid hormone receptors and hormonal response, presumably via neuronal pathways. The odor of male prairie voles induces estrous behavior in female prairie voles,[404] presumably in part due to the accompanying increase in the concentration of ERs in the mPOA.[405] Level of maternal care of pups induces long-term changes in the concentration of ERα in particular brain areas,[406] due to epigenetic changes (see Chapter 52) in the ERα gene promoter.[407] Likewise OT, injected systemically during the neonatal period, induces stable changes in the levels of ERα in prairie voles.[408,409] Therefore, regulation of steroid receptors by environmental stimuli working through neurotransmitters is another level of regulation of steroid hormone response in subsets of relevant neurons.

Ligand-Independent Activation of PRs

Steroid hormone receptors, such as ERs and PRs in addition to being activated by binding of their cognate ligand, steroid hormone receptors can be activated by a variety of intracellular signaling pathways.[410,411] Power et al.[412] first demonstrated in vitro that DA agonists also can activate PRs in vitro, which led to studies on alternate pathways to PR activation besides binding of progesterone, including pathways leading to the facilitation of sexual behavior in the absence of progesterone.

Intracerebroventricular infusion of D1 DA agonists substitute for progesterone in the facilitation of sexual behavior in estradiol-primed rats.[413] This facilitation by DA agonists is blocked by infusion of progesterone antagonists,[413] or antisense oligonucleotides directed at the PR mRNA,[369] or in PRKO mice,[373] providing strong evidence that DA facilitates the expression of sexual behavior by indirectly activating PRs in the brain in vivo.

Progesterone and DA both initiate second messenger signaling cascades involving increases in 3′-5′-cyclic adenosine mono phosphate (cAMP) levels, activation of PKA and phosphorylation of the neuronal phosphoprotein, DA and cAMP regulated phosphoprotein-32 (DARPP-32)[414,415] (Figure 50.14). These in turn result in alterations in phosphorylation of other proteins and activation of PRs and/or its coregulators in the hypothalamus. DARPP-32 KO mice express a decreased level of female sexual behavior in response to either progesterone or DA. This suggests that DARPP-32 is involved in ligand-independent activation of sexual behavior via PRs.[414] These results demonstrate the obligatory role that activation of DARPP-32 plays in the regulation of sexual receptivity by PRs, regardless of the route of activation of the receptors.

The mechanism of ligand-independent activation of steroid receptors by neurotransmitters and second messenger pathways provides a possible means by which afferent input from the male facilitates the expression

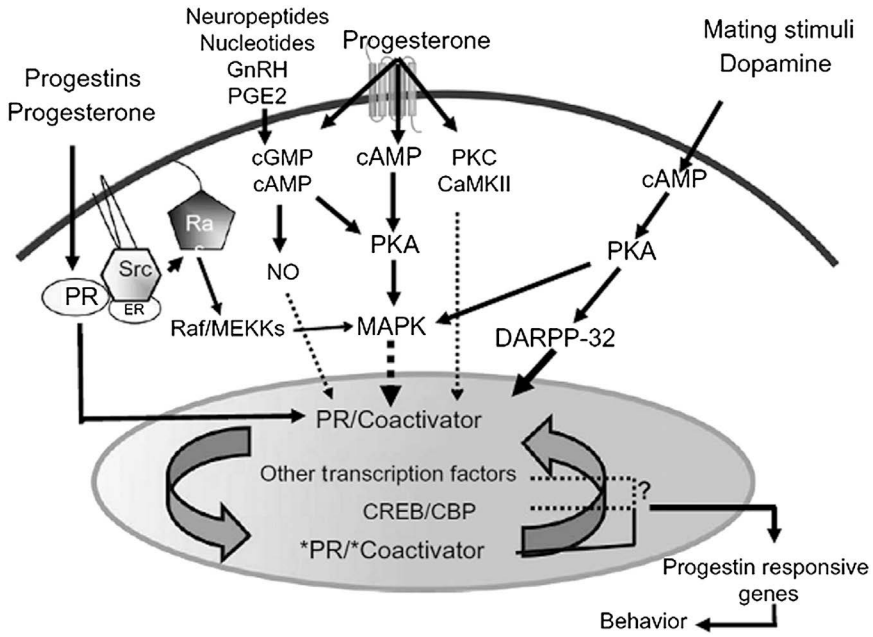

FIGURE 50.14 Cross-talk between progesterone and a variety of second messenger pathways converging on the PR leading to increases in female sexual behaviors. This schematic representation depicts a variety of interacting mechanisms. (1) Progesterone acts via a classic genomic mechanism of action mediated by classic (primarily) nuclear PRs. The ligands bind to their cognate receptors and activate PRs promoting interactions with coactivators; (2) Progesterone acts via second messengers (cAMP, cGMP) and signaling kinases (PKA, PKC, CaMKII), which then activate the MAPK signal transduction cascade, leading to phosphorylation of PRs and coactivators (CREB and/or its associated protein CBP shown here, as well as others); (3) Progesterone and progestins act via the Src kinase pathway to activate the MAPK cascade leading to activation of PR and coactivators. (4) Progesterone acts via the PKA/MAPK/DARPP-32 pathway to induce an increase in phosphorylation of PRs and/or its coactivators. (5) Mating stimuli and D1 agonists may stimulate PKA activation, which then phosphorylates DARPP-32 or MAPK leading to the activation of PR and/or its coactivators. (6) Neuropeptides, nucleotides, prostaglandin E2, and other molecules may act through various receptor- and/or second messenger pathways (cAMP, cGMP, NO) to activate nuclear PRs or other transcription factors. *PR/*Coactivator signifies activated progestin receptor and coactivators. Dashed lines indicate hypothesized, but as yet unproven, direct interactions. *Source: Reprinted from Mani and Portillo,[415] with permission of Elsevier.*

of sexual receptivity in the absence of progesterone. First some background information is necessary. When estradiol-injected, OVX or OVX/ADX rats are exposed intermittently to sexually active males, their level of sexual receptivity increases over the first few hours,[179–181,416–419] even if the ovaries and adrenal glands are both removed, presumably removing all sources of peripheral progesterone.[418] Progestin antagonists block mating-enhancement,[418] suggesting that the cellular mechanism by which the male's mating attempts facilitates sexual behavior is via ligand-independent activation of PRs.[418] It should also be noted that mating-related stimulation results in phosphorylation of DARPP-32 in areas associated with reproduction and reproductive behavior (mPOA, VMH, posterodorsal MEA, and BNST),[420] and a D1 DA antagonist eliminates Fos expression in response to mating-related stimulation in all areas investigated.[421] Although the evidence supports ligand-independent activation of PRs as the mechanism for mating-enhancement of sexual behavior, the possibility that neuroprogesterone is involved cannot be excluded at this time.[422]

Because sexual stimulation induces DA release,[423–426] immediate early gene response (Fos) in PR-containing neurons,[427] and DARPP-32 phosphorylation in relevant areas,[420] it is likely that mating-induced DA activates PRs via ligand-independent activation mechanism. The PR-dependent, ligand-independent relationship is involved in the mechanism by which a variety of compounds that substitute for progesterone facilitates sexual behavior. GnRH,[428] delta opioid receptors,[429] α_1-adrenergic receptors,[430] nitric oxide (NO),[431] MAP kinase,[429] PKA,[432] cAMP,[428] and cGMP[433] as well as prostaglandin E2[428] and epidermal growth factor[434] each facilitate sexual receptivity in estradiol-primed rats by a process that involves activation of PRs. Likewise, ligand-independent activation of PRs may be involved in the mechanism by which methamphetamine enhances the effects of ovarian hormones on female sexual behavior.[435] Thus, many of the numerous signaling pathways that induce female sexual behavior in estradiol-primed rats converge on ligand-independent activation of PRs.

Ring-A reduced metabolites of progesterone, 5α-dihydroprogesterone and allopregnanolone, as well as 5β-reduced progestins, in addition to GnRH, and prostaglandin E2, facilitate lordosis in OVX, estradiol-primed female rats.[436–440] Because these progestins and

hormones have relatively low or no affinity for PRs, it is unlikely that they facilitate lordosis by binding directly to PRs. However, facilitation of sexual behavior by these compounds is inhibited by a progestin antagonist, suggesting involvement of ligand-independent activation of PRs in the process by which they facilitate sexual behavior.[432,437,441] Further, it has been suggested that via ligand-independent activation of PRs, they, like progesterone,[442] then activate the Src/Raf/MAPK signaling pathway resulting in lordosis.[440] Therefore, even some progestins may facilitate the expression of sexual receptivity by ligand independent activation of PRs after which they converge on a signaling pathway in common with a pathway involved in facilitation by progesterone.

As mentioned earlier, progesterone facilitation of sexual behavior is dependent on the PR-A isoform. In contrast, ligand-independent activation of sexual behavior by a D1 DA agonist is reduced, but not eliminated in either PR-A or PR-B KO strains of mice.[374] Therefore ligand independent activation of sexual behavior is dependent on both isoforms. Sexual behavior facilitated by the PKA activator, 8-bromo-cAMP, is eliminated in PR-A KO mice. Therefore, each isoform is involved in the facilitation of sexual behavior, but the importance of each isoform may depend on the mode of activation. Although PR-A seems to have the primary role in most situations, PR-B has a larger role in ligand-independent activation of sexual behavior.

The fact that many neurotransmitters can both influence steroid receptor levels and activate steroid receptors raises questions in the interpretation of many pharmacological studies. Pharmacological experiments performed with the assumption that the drugs are stimulating or antagonizing output of steroid hormone-sensitive neurons. However, it is likely that some of the drugs are influencing steroid receptor-containing neurons, either activating steroid hormone receptors directly or regulating the concentration of receptors.

Ultrastructural Changes Induced by Estradiol and Progesterone

Hormonal Regulation of VMH Structure

The VMH is well established as a key site in the control of female sexual behavior by ovarian steroid hormones, as it has been extensively studied in rats, as discussed above. It should be noted that the borders of the VMH as defined by Nissl stain may not perfectly match the distribution of typical VMH markers, such as ERs, in some species. Nevertheless, the term "VMH" is useful as referring to a brain region with functional similarities across vertebrates, even if its borders and connectivity differ somewhat in various species.

Hormone-induced changes in VMH structure must be understood within the context of the VMH local circuit, which comprises several cell types. Unfortunately, phenotypic markers for these cell types are only partially defined (Table 50.2), and the direction of information flow between these cell types is not yet known. One cell type expresses nuclear ERα and estradiol-induced PR. Given that many studies have used nuclear labeling to identify the ERα-expressing cells, it remains unclear whether these same neurons also mediate the membrane-based effects of estradiol discussed above. Given that these neurons express OT receptors, their long dendrites manifest an estradiol-induced increase in synapses, as discussed below.

A second cell type, are neurons with axonal connections to the periaqueductal gray,[443,444] a critical relay in the control of the lordosis reflex. A small subset of PAG-projecting neurons expresses steroid receptors (15–25%). These neurons have a more elaborate dendritic tree, and estradiol treatment reduces spines on the long dendrites of these neurons.

A third cell type within the VMH is defined in part by the lack of ERα and axonal projections to the midbrain, and the presence of mating-induced Fos expression[427,444,445] These neurons exhibit a robust estradiol-induced increase in dendritic spines. In summary, the VMH microcircuit includes at least three cell types that exhibit differential patterns of neural plasticity after estradiol treatment.

Recent insights into the ovarian hormone-induced remodeling of neural circuits within the VMH in adult female rats provide insights into the neurological control of this behavior. It is worth noting that estradiol-induced synaptic changes have been observed in a number of

TABLE 50.2 Phenotypic Markers of Three Types of Neurons in the VMH Local Circuit

Cell Type	Features
ER/PR expressing neurons	Glutamatergic Co-express enkephalin Dendrite arbor not known Weakly activated by mating Express oxytocin receptors Estradiol increases synapses on LPDs
PAG projecting	Transmitter not known Five to six dendrites Weakly activated by mating Estradiol reduces LPD spines (length)
"Unidentified" (lack ER/PR and no projections to the PAG)	Transmitter not known Three dendrites Activated by mating Estradiol increases spines on short dendrites Estradiol reduces LPD length

other brain systems that are not directly tied to sexual behavior. These include the hippocampus[446] and the PFC.[447] Thus, it seems that estradiol's effect on synaptic organization in the VMH represents a typical mode of action for this hormone.

Estradiol has global structural effects on neurons in the VMH. Hours after OVX animals are treated with estradiol the size of neuronal cell bodies is increased, based in part on the hypertrophy of the nucleus, rough endoplasmic reticulum, and Golgi apparatus.[448] In addition, estradiol changes several parameters within the dendritic arbor (Figure 50.15). The size and shape of a dendritic tree are indicative of its ability to sample and weight afferent inputs. Neurons in the VMH exhibit

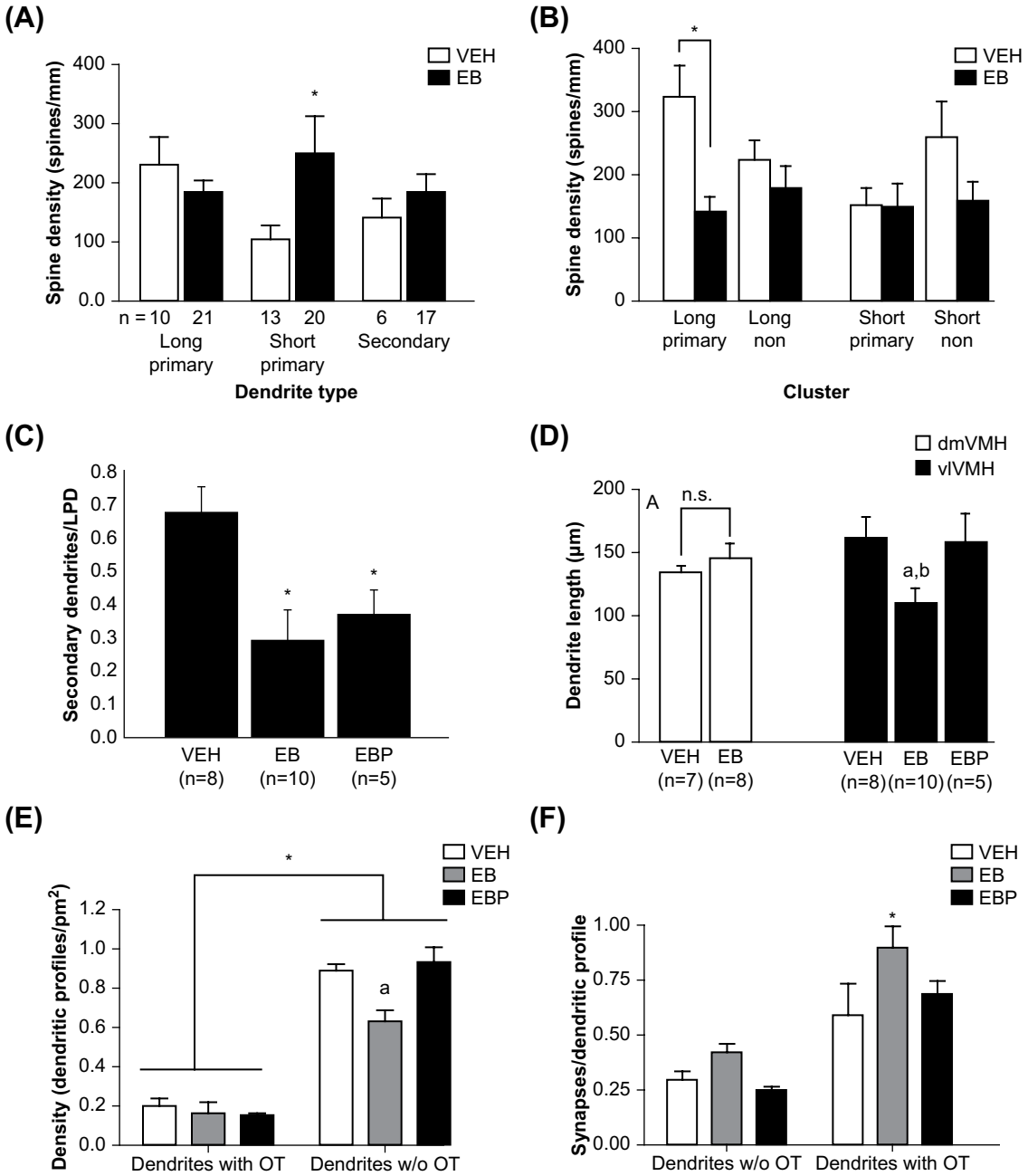

FIGURE 50.15 Complex effects of vehicle (VEH), estradiol (EB), or EB and progesterone (EBP) on the dendritic arbor of VMH neurons. Golgi impregnation and electron microscopy studies show that estradiol increases spines on short primary dendrites (Panel A); reduces dendritic spines on long primary dendrites of projection neurons (Panel B); decreases branches on long primary dendrites (LPD) (Panel C); retracts long primary dendrites (Panel D); selectively removes oxytocin (OT)-negative dendrites from the lateral fiber plexus (Panel E); and increases innervation on oxytocin-positive dendrites in lateral fiber complex (Panel F). *(Source: Adapted from Calizo and Flanagan-Cato,[443] Calizo and Flanagan-Cato,[449] Griffin and Flanagan-Cato,[450] and Griffin et al.[451])* vlVMH, ventrolateral VMH; dlVMH, dorsolateral VMH.

simple dendritic arbors, usually with three or four dendrites. Based on studies using various techniques to quantify neuronal morphology in intact estrous cycling rats and OVX females given ovarian hormone replacement, the number of dendritic spines correlates with hormonal states that increase reproductive behavior.[449,452–454] Thus, estradiol treatment increases local excitatory input onto VMH dendrites (Figure 50.16).

At the same time, long dendrites retract from the lateral afferent zone, a process that is reversed with subsequent progesterone treatment. This regulation of VMH dendrite length was first observed with Golgi analysis[450] and subsequently confirmed with electron microscopy analysis.[451] Ultrastructural evidence suggests that non-OT receptor-bearing dendrites retract, allowing for the transfer of afferent inputs to the OT-bearing dendrites.[455] Thus, estradiol treatment appears to simultaneously heighten local excitatory input and direct extranuclear afferent input to the OT-sensitive neurons, suggesting a substantial hormone-dependent shift in the computational activities of VMH neurons and their outputs that control sexual behavior (Figure 50.16, bottom).

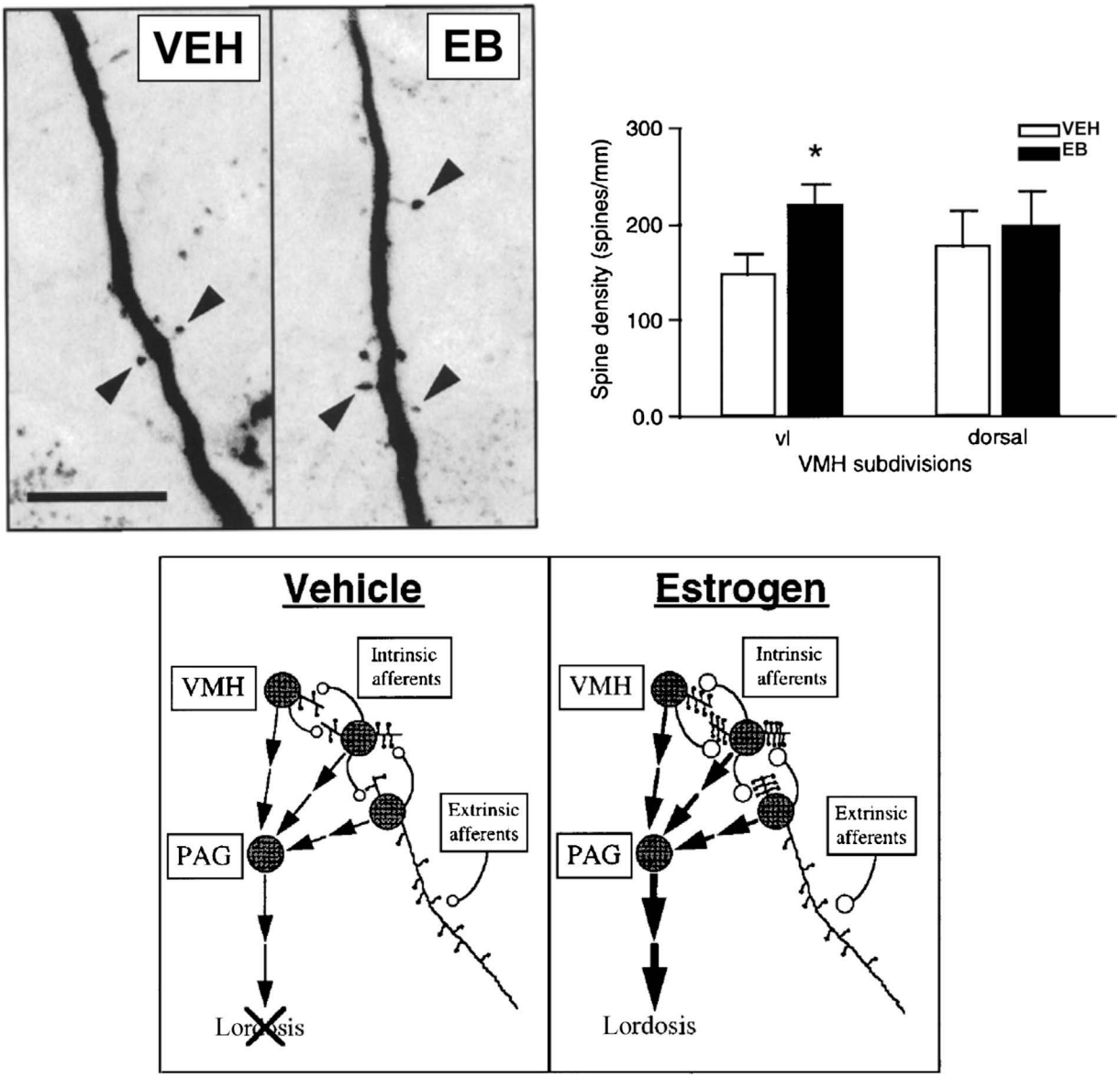

FIGURE 50.16 Top left: Confocal projections of representative female rat VMH dendrites illustrating the effect of estradiol treatment to increase the number of spines. Arrows indicate the locations of representative spines. Scale bar = 10 μm. Abbreviations: VEH, vehicle; EB, estradiol benzoate. Top right: Mean spine densities from vehicle- and EB-treated rats in the ventrolateral (vl) and dorsal regions of the VMH. *$p < 0.05$. Bottom: Model of possible mechanisms of spine induction by estrogen on short primary dendrites in the VMHvl. The output of the increased action of intrinsic afferents would be to increase the activation of the periaqueductal gray (PAG) to reach a critical threshold for lordosis to occur. *Source: Reprinted from Calizo and Flanagan-Cato,[449] with permission from the Society for Neuroscience.*

An important projection from the VMH terminates in the ventrolateral PAG,[456] a critical relay in the motor control of lordosis behavior. Estradiol treatment induced remodeling of synaptic organization in this region as well.[457] In particular, estradiol treatment increases the number of synapses and the number of dense-core vesicles per synapse. As mentioned above, ERα is abundant in the PAG. To date, little is known about hormone-induced plasticity in the medullary reticular formation or spinal motor neurons, although in the cat estradiol increases the innervation of the nucleus retroambiguus to the lumbar spinal cord.[458] It would not be surprising if dendrite structure in the lumbar ventral horn were also changed. Thus, ovarian hormones may promote lordosis by modifying the connections between several nodes in the descending lordosis pathway.

The relevance of synaptic reorganization of the VMH in human sexual behavior has been not been studied due to obvious technical and ethical limitations. However, hormone-induced regulation of VMH dendrite length has been documented in other rodents, namely hamsters[459] and prairie voles,[460] which indicates that it is not a phenomenon unique to rats. Studies in nonhuman primates would be helpful to assess the possible relevance to sexual behavior in women. The synaptic reorganization that has been observed with structural studies is associated with altered activity of VMH neurons (discussed below).

Genitosensory Stimulation Activates Cells with Steroid Hormone Receptors

Many of the cells that respond to mating-related stimulation also contain ERα-ir[444,445] and/or PR-ir[427] suggesting that they are part of the neuronal substrate for integration of hormonal signals with afferent input from the social environment. Mating- or VCS-induced Fos is extensively coexpressed with ERα-ir in the mPOA, bed nucleus of stria terminalis, posterodorsal MEA, midbrain central gray[445] and VMH.[444,445]

Although other areas were not investigated, extensive coexpression of Fos with PR-ir is seen in the mPOA, parts of the VMH, and the ArcN.[427,461] Within the VMH (primarily rostral), mating induces Fos-ir in a number of PR-ir cells that project to the midbrain central gray[462] consistent with a role for these neurons in female sexual behavior. The fact that they are responsive to mating stimulation and express PRs suggests that they may be a substrate for interaction between mating stimulation and PRs.

In some, but not all cells, VCS-induced Fos expression in estradiol-primed, OVX rats requires PRs, suggesting that ligand-independent activation of PRs is an obligatory step in the activation of the c-fos gene.[418,461] Although progestin antagonists block VCS-induced Fos expression in the mPOA, medial bed nucleus of stria terminalis, and caudal VMH, they were without effect in other areas studied, including the MEA, dorsomedial hypothalamus and PVN. Interestingly, a progestin antagonist blocks VCS-induced Fos expression in the rostral, but not caudal, mPOA.[461] Collectively, the data support the interpretation that in cells expressing PRs, VCS induction of Fos expression requires functional PRs; that is to say, neuronal response to particular stimuli seems to be gated by PRs. The mechanism is likely to be ligand independent activation of those PRs by the afferent input. Although olfactory stimuli are of great importance to reproduction and sexual behavior, unlike VCS-induced Fos expression[427] the neurons in which exposure to bedding soiled by male rats induces Fos expression do not contain PR-ir.[463]

In a study of responses of ERα and ERβ cells to mounts with and without intromission, Gréco et al.[464] reported that either mounts alone or mounts with intromissions induced Fos in ERα-ir cells within the rostral mPOA. However, Fos expression in ERα/ERβ coexpressing cells was induced only by mounts with intromission. The pattern in the amygdala was different. In the dorsal posterodorsal MEA, only mounts that included intromission induced Fos expression, and it did so only in ERα or in ERα/ERβ coexpressing cells, not in cells expressing only ERβ. In the ventral posterodorsal MEA, only mounts with intromission induced Fos expression, but the response was restricted to ERβ cells, but not cells expressing ERα. Although work in ERKO mice suggests primary involvement of ERα in female sexual behavior, these results suggest roles for both forms of ERs in integration of hormonal information and information relating to specific types of sexual or genital stimulation.

ARs and Aromatase

Androgens, principally testosterone and 5α-DHT, exert their effects by activating a specific AR. Thus far, a single subtype of AR has been described, a classic member of the steroid receptor superfamily, a hormone-activated transcription factor, with the canonical functional domains of proteins in this superfamily. Thus, upon ligand binding, AR undergoes phosphorylation, homodimerizes, interacts with DNA, and binds to androgen response elements. Transcriptional machinery and cofactors are then recruited to the site to promote or repress gene expression. In addition to this standard genomic mechanism, AR is found in dendrites and axons, suggesting short-term effects on neurotransmission.[465,466] Some brain regions respond to testosterone by converting it to estradiol, via the enzyme aromatase, also known as cytochrome P450 enzyme. Aromatase is found in the smooth endoplasmic reticulum of some populations of neurons. Thus, aromatase activity would direct

TABLE 50.3 Brain Regions Involved in Female Sexual Behavior that Contain Dominant ER Subtypes, AR, and/or Aromatase Expression

	ER-Alpha	ER-Beta	Both	AR	Aromatase
Amygdala	Amygdalohippocampal area		Medial nuclei	Medial nuclei	Medial nuclei
			Cortical nuclei	Cortical nuclei	Cortical nuclei
Septum	Subfornical organ		BNST	BNST	BNST
				Lateral septum	
Hypothalamus	mPOA	PVN	mPOA	mPOA	mPOA
	AVPV				
	ARC			ARC	
				PVN	PVN
	VMH			VMH	VMH

circulating testosterone to act via ERs rather than AR. Therefore, the expression of both AR and aromatase will be considered for a full view of testosterone action in the female brain.

Multiple techniques and animal models have helped describe the distribution of ARs in the vertebrate brain, including rats, mice, hamsters, and nonhuman primates; however, there is surprisingly limited information focused on adult female mammals. Available evidence suggests that females and males have similar neuroanatomical distributions of AR, with somewhat different levels in females. Table 50.3 includes a listing of the brain regions with the highest levels of ARs, based on these studies.

The first studies delineating the anatomical distribution of testosterone binding used steroid receptor autoradiography with 3H-testosterone injected systemically. Abundant testosterone binding was reported in the preoptic area, amygdala, and especially the VMH, in female rats[467,468] and female rhesus monkeys.[469] The molecular cloning of AR led to the development of antibodies that could be used in immunocytochemical procedures, although initial reagents were plagued with conflicting results. The messenger RNA for AR has been mapped with in situ hybridization techniques,[269] and these results nicely complement the distribution described with autoradiography. Studies in juvenile females indicate that AR mRNA is abundant in several brain regions associated with female reproductive behavior, including the lateral septum, BNST, mPOA, and VMH.[470] Although it has not been studied extensively, it appears that in some brain regions that co-express AR and ER, many neurons express AR only, but most neurons that express ER co-express AR.[471]

As with AR, most studies on aromatase in the brain have focused on developing males rather than adult females, but some basic information exists. Aromatase has been documented with in vitro enzyme activity assays of discrete brain regions, including the BNST, mPOA, MEA, and VMH.[472] This analysis also compared the levels of aromatase and AR in adult females. A striking finding was that AR binding activity is highest in the VMH, where aromatase activity is the lowest. The relatively low level of aromatase activity in the VMH is reflected in relatively low levels of aromatase messenger RNA.[473] This finding suggests that in the VMH testosterone may be more likely to act through the AR than the ER in females. Ovarian hormones do not regulate aromatase activity in female rat brains.[474] Overall, transcripts for aromatase are found in the BNST, the mPOA, the MEA, and the VMH in females.[475]

In summary, ARs are expressed in the female brain, including in brain regions that promote female sexual behavior. Although some brain regions may convert testosterone to estradiol, androgen-specific actions that affect female sexual behavior seem likely, especially in the VMH. Although not well studied yet, it is possible that 5-alpha reductase is also expressed in the VMH in female mammals, as it is in green anole lizards.[476] This enzyme converts testosterone to the more potent androgen, DHT. If so, females would have the potential to compensate for their relatively low circulating levels of testosterone by locally enhancing androgen action. Although there is little information about the role of androgens in female sexual behavior, ARs appear to be poised to contribute.

Hormone Replacement Therapy in Women

A decline in sexual arousal, desire, and/or activity occurs following surgical and natural menopause. Surgically menopausal women, induced by bilateral oophorectomy with or without hysterectomy experience a sudden and drastic decline in sexual desire and arousal.[126–129] Significant improvement in desire and arousal occur following adequate hormone replacement regimens,

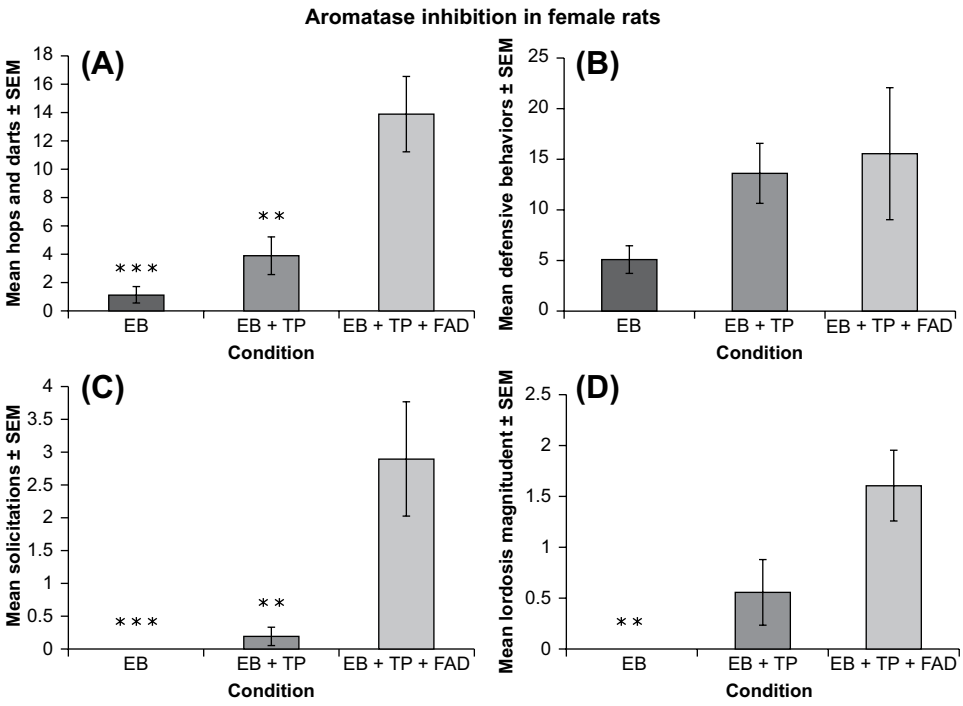

FIGURE 50.17 Hops and darts (A), solicitations (C), defensive responses (B), and lordosis reflex magnitudes (D) of OVX rats primed with estradiol benzoate (EB) alone, EB + testosterone propionate (TP), or the combination of EB + TP along with the aromatase inhibitor fadrazole to block conversion of testosterone to estradiol, thus augmenting the effect of the exogenously-administered TP on behavior. *(Source: From Rosenbaum, Jones, and Pfaus.[478])* $^{**}p < 0.05$, $^{***}p < 0.01$, from the EB + TP + FAD-treated rats.

particularly replacement with exogenous estrogens in combination with testosterone[126,127,131,133,135–139] relative to estrogen treatments alone. Although co-administration of estrogens and progestins (notably medroxyprogesterone acetate) also increased sexual arousal and desire, this formulation increased overall risk for secondary disease factors in the Women's Health Initiative randomized trials.[477]

An unresolved question is how co-therapy with estrogens and androgens works and why it offers greater therapeutic value over estrogen replacement alone. There are at least three possible scenarios. The first is that the induction of SHBGs by exogenous estrogens would likely decrease free estrogen concentrations, thereby limiting their ameliorative action in the brain and periphery. The addition of androgens like testosterone to this would compensate by binding to SHBGs with higher affinity, thus allowing more free estrogens to get to target tissues. The second possibility is that testosterone induces actions on its own through binding to ARs in the brain and periphery. Indeed, a significant proportion of untreated postmenopausal women reported increases in sexual desire and activity following double-blind administration of testosterone for 6 months through a transdermal patch that released approximately 300 μg/day of testostereone.[137] This treatment increased free testosterone to within normal physiological premenopausal levels without any concomitant increases in plasma estradiol or SHBG levels. Placebo control conditions did not induce this increase. These data suggest that testosterone alone is able to reverse the decline in sexual arousal and behavior in postmenopausal women. However, the presence of aromatase in the brain could convert exogenous testosterone into estradiol locally in different hypothalamic or midbrain regions, despite no increases in plasma estrogens. Long-term OVX rats treated systemically with the aromatase inhibitor fadrozole and given hormone replacement with the combination of estradiol and testosterone displayed significantly greater numbers of appetitive solicitations and lordosis responses compared to females given the combination of estradiol and testosterone alone (Figure 50.17). Females treated with estradiol alone displayed low numbers of solicitations and lordosis responses.[478] Thus, blocking the aromatization of testosterone into estradiol induces a greater facilitation by testosterone of both appetitive and consummatory aspects of female sexual behavior relative to estradiol alone or the combination of estradiol and testosterone. This suggests strongly that testosterone acting at ARs facilitates female sexual behavior. The final possibility of course is that both testosterone and estradiol exert distinct actions on ARs and ERs, respectively, that combine to facilitate sexual desire and behavior.

NEURAL ORGANIZATION

As mentioned above, sexual behavior has a beginning, middle, and end, each of which is controlled by different, but integrated, brain systems. The function of many of those systems is altered by steroid hormones, such that sexual incentive stimuli come to the foreground

of attention and action. These neural systems must be able to process rudimentary sensory components of sexual stimulation (e.g., olfactory, visual, somatosensory, auditory), and sum them into Gestalts or "wholes" that represent contextual and/or discrete unconditioned incentives (e.g., suitable receptive partners, partner odors, individual facial features, etc.) or unconditioned inhibitors (nonreceptive partners, predators, or parents, or competing incentives like food if hungry), and do the same for conditional incentives or inhibitors (e.g., neutral cues that become predictors of the unconditioned incentives, e.g., a favorite sex toy, odor or place cue associated with sexual reward, or inhibitors, e.g., same stimuli associated with thwarted sexual behavior or sexual nonreward). They must be able to compute competent responses to those cues so that sexual behavior is timed correctly and appropriate to the circumstances, and automate those responses so that they become more optimal with experience (e.g., faster initiation of solicitations in the presence of highly valued partner cues). At each level of analysis, there are excitatory and inhibitory neurochemical systems that regulate the intensity of the perceived incentive or inhibitory stimuli and that modulate the timing and intensity of the responses.[35] These are also modulated by steroid hormones and by feedback from positive and negative sexual experiences (see Section Consequences of Sexual Stimulation).

Excitation, Inhibition, and Disinhibition of Sexual Responses

The notion of separate, but interactive, neural systems for behavioral excitation and inhibition (Figure 50.18) goes back to the work of early neurophysiologists like Sechenov, Sherrington, and Pavlov, and more modern psychologists like Gray, who applied the idea to the study of fear and anxiety.[480] It has important implications for sexual behavior because it posits that behavior can commence either due to direct excitation or through a process of disinhibition. This concept was advanced further by Bancroft and Janssen[481] and Perelman,[479] who presented dual control models of human sexual response in which the net expression of sexual behavior is based on the influence of excitatory and inhibitory mechanisms in the brain and periphery, set around a "sexual tipping point". As in Gray's theory, this model stressed the adaptive nature of both excitatory and inhibitory processes. For example, the adaptive nature of sexual excitement would drive individuals to seek out sex partners for reproductive or reward purposes. The adaptive nature of sexual inhibition would guard against situations that threaten the individual, including chronically stressful life events. It would also be important to keep the optimal expression of behavior constrained to the "right time", as in the case of females that display sexual behavior only during a periovulatory period, when they are most likely to become pregnant. Bancroft and Janssen viewed the propensity for sexual excitement or inhibition as an individual tendency based on the genetic makeup and/or behavioral expectations of the individual: Those whose propensity for central inhibition of sexual response is too high have increased vulnerability to sexual dysfunction, whereas those whose inhibitory propensity is too low would be more likely to engage in hypersexual or high risk sexual behavior. Indeed, the study of sexual inhibition is also critical if we are to understand how certain events or drugs like alcohol, cocaine, or amphetamine, may induce sexual disinhibition and the propensity to engage in risky sexual behaviors.[482]

Excitation

We may view excitation from autonomic arousal and genitosensory/erogenous stimulation as a "bottom-up" phenomenon in which individual sensory modules come together at higher levels of processing. This occurs in the thalamus, but also in each domain of the hypothalamus, limbic, system, and cortex, that make up the excitatory sexual system of the brain.[35] At the hypothalamic level, genitosensory and olfactory information is integrated in both the mPOA and VMH, which have outputs to the PVN, SON, and ArcN of the hypothalamus. In turn, those regions control the release of OT, vasopressin both in brain and from the posterior pituitary, and the release of melanocortins (MCs), opioids, and corticotrophin releasing factor (CRF) from the anterior pituitary. The mPOA also sends lateral efferents to the VTA, which stimulate DA neurons and DA release in mesolimbic and mesocortical terminals, such as the NAc, ACC, lateral septum, corticomedial amygdala, and mPFC. Thus, the mPOA is well situated to "drive" the DA-mediated incentive motivational system in the presence of salient unconditional and conditional external sexual cues, and also to register and perhaps link those cues to genitosensory and autonomic input. In addition, noradrenergic inputs to the hypothalamus coming from the locus coeruleus are themselves stimulated by the reticular activating system, which is stimulated by general autonomic and somatic inputs from the spinal cord. For females, sensitivity to sexual arousal is enhanced during ovulation, and thus excitatory systems in the CNS appear to require steroid hormone priming.

Estradiol regulates a variety of neurotransmitter and second messenger systems in brain areas involved in sexual behavior.[483,484] Estradiol priming induces the synthesis of α_{1B}-adrenergic receptors in the VMH[485,486] and augments the release of DA in the striatum, NAc, and mPOA during copulation.[90,425,487,488] Extracellular DOPAC (3,4-dihydroxyphenylacetic acid), a DA metabolite, increases in the mPOA in the afternoon to the early evening of proestrus, around the time that sexual

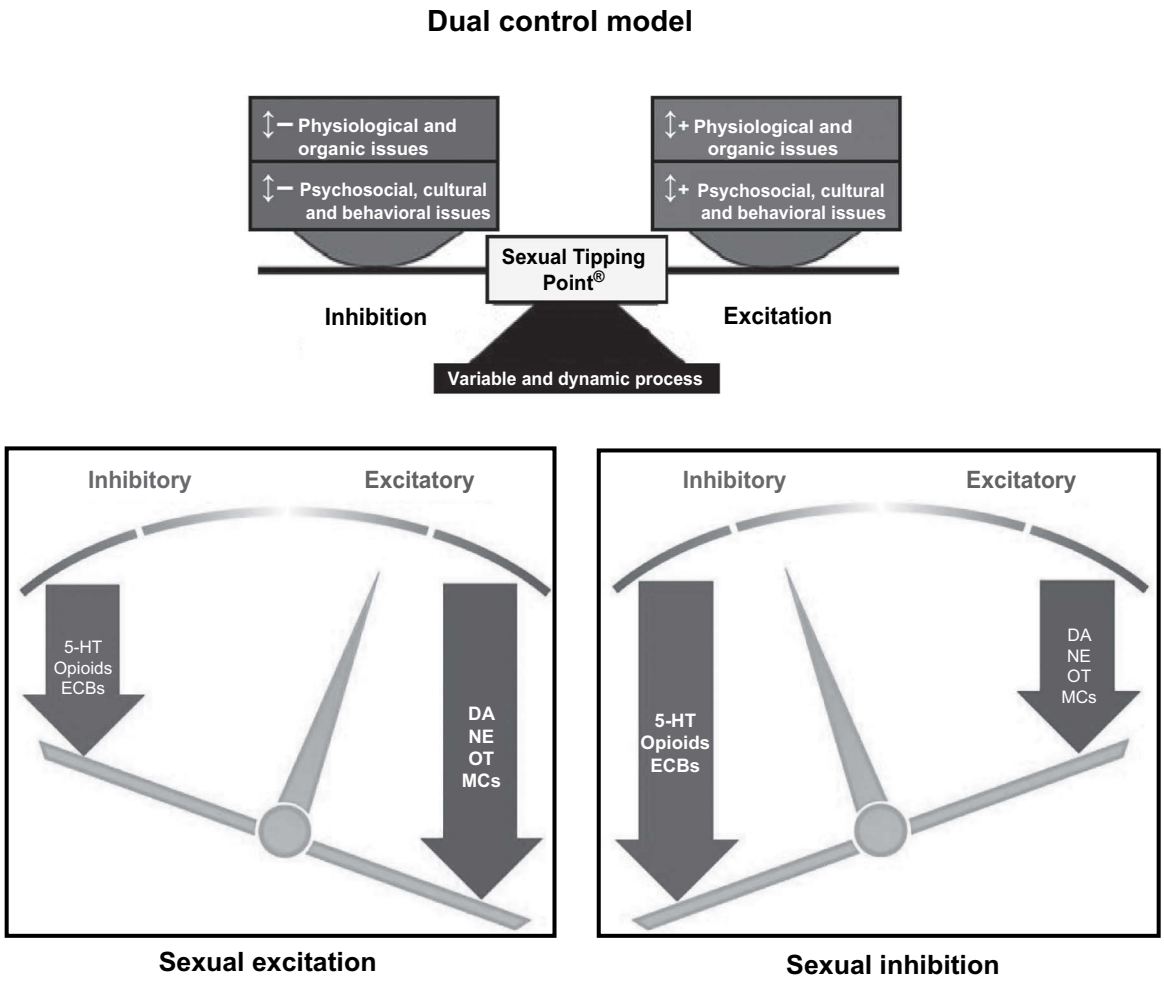

FIGURE 50.18 **Dual control model of sexual excitation and inhibition around a "sexual tipping point".** Activation of excitatory neurotransmitters, such as dopamine (DA), norepinephrine (NE), oxytocin (OT), and melanocortins (MCs), within midbrain, hypothalamic, and limbic regions occurs in response to steroid hormones, sexual incentive cues, and sexual stimulation. Activation of inhibitory neurotransmitters, such as serotonin (5-HT), opioids, and endocannabinoids (ECBs) in cortical, limbic, hypothalamic, and brainstem regions occurs during and after orgasm or in response to stress or aversion, resulting in a net decrease in excitatory tone. *Source: Adapted from Perelman*[479] *and Pfaus.*[35]

behavior is activated.[482,489] DA is also released within the mPOA in OVX females treated with EB and progesterone, however this release is not detected in females treated with EB alone.[424,490] Hormone dependent differences also occur with the application of DA agonists to the mPOA, such that in females primed with EB-alone, D2 receptor subtype agonists facilitate sexual behavior, whereas in EB + progesterone primed females D1 receptor subtype activation facilitates sexual behavior.[482,491,492] Tonic DA release activates D2 receptors, whereas phasic DA release stimulates D1 receptors. Thus, priming with EB-alone may tonically activate D2 receptors, whereas subsequent progesterone administration may stimulate phasic DA release within the mPOA, and activate D1 receptors, to facilitate sexual behaviors. Estradiol also stimulates the synthesis of MC type four receptors in the hypothalamus[493] and synthesis of proopiomelanocortin (POMC) in ArcN neurons[494] and stimulates the synthesis of cholinergic receptors and enkephalin within the VMH.[495,496] GABA and glutamate activation are also stimulated by estradiol in these regions (see below).

Inhibition

Inhibitory synapses make up a large part of the CNS, and local inhibitory networks can hone a response by eliminating competing responses that would interfere with it (e.g., as happens in the visual system with lateral inhibition by amacrine cells[497]). Such inhibition can be observed when behavior comes in bouts or phases, or in Masters and Johnson's EPOR model would be consonant with the "R" phase, a period of postorgasmic refractoriness in which further sexual interest is diminished.[21] Local inhibition can also play a role in the timing of behavior to make it occur only during optimal periods. Female desire and lordosis behavior are obvious examples, and the role of steroid hormones may well be

to suppress the behavior during reproductively nonoptimal (nonovulatory) periods.

General behavioral inhibition, however, is typically viewed as a "top-down" phenomenon involving the cognitive process of "executive function".[498] Animals always have to choose among different drives and motivations (e.g., between feeding and copulation), and indeed between several possibilities per motivational system, to achieve an optimal outcome. The mPFC organizes this by creating behavioral hierarchies based on expectancies, planned actions, and calculations. The mPFC, (and likely other cortical areas) therefore, must inhibit a complex and ongoing interplay of motor tendencies to arrive at planned and sustained actions. People or rats with disrupted prefrontal function, either due to lesions or neurochemical imbalance, have great difficulty focusing attention on tasks, are unable to inhibit competing responses, and experience retroactive and proactive interference.[498] With regard to sexual behavior, such top-down inhibition can be activated as "morality" and would be based on a cultural value system that imposes "right" and "wrong" on certain behaviors, such that some that feel good are "right" and can be experienced without guilt, whereas others are "wrong" and carry the weight of guilt and/or rule of law against them.[28,35] This type of inhibition gives rise to the classic "approach-avoidance" conflict, where the expectation of reward drives the desire, but the inhibition imposed by the real or perceived aversive consequences of engaging in sexual activity blunts the initiation of behavior. Such inhibition may well lie at the root of the inhibited sexual response experienced by women who are not taught to express their sexual desires without some form of guilt. Such inhibition would likely be reinforced if women experienced sexual nonreward during copulation, and such reinforced inhibition would likely overlay itself on desire components to suppress them directly. Some women may be more susceptible to this type of inhibition than others. Accordingly, the "prosexual" nature of drugs such as alcohol, cocaine, and methamphetamine may be a function of their ability to disinhibit such suppressed sexual responding.

Refractory inhibition that comes after orgasm involves the activation of at least three neurochemical systems: opioids that induce pleasure, euphoria, and ecstasy; endocannabinoids that induce sedation; and serotonin that induces satiety.[35] The reward states induced by CLS, VCS, or paced mating in rats appear to be independent of steroid hormone priming, although the priming is necessary to activate the excitatory systems that bring about the behavior in the first place.[28,162] At present it is not known where such inhibition actually takes place. Indeed, the mPOA and VMH have receptors for all three transmitter systems, indeed for all three opioid systems. Activation of delta opioid receptors in the mPOA inhibit lordosis,[499] whereas activation of mu opioid receptors in the VMH inhibits lordosis.[485] Inhibition also comes in the form of estrus termination. This is discussed in more detail below.

Disinhibition

As noted above, certain prosexual drugs can disinhibit sexual responding, but only in individuals with sexual inhibition. This has been modeled in male rats.[90] Male rats trained not to copulate with sexually nonreceptive females will attempt copulation with them under the influence of alcohol. Cocaine, amphetamine, and methamphetamine can do the same in males,[482] and Frohmader et al.[490] provided evidence that methamphetamine could do this after males were inhibited from making sexual approach responses toward scented, sexually receptive females using LiCl-induced gastrointestinal distress as the inhibitory outcome. Alcohol and cocaine stimulate appetitive behaviors in females primed with estradiol alone.[482] Intermittent amphetamine or methamphetamine administration sensitizes sexual approaches and solicitations in female rats[435,500] and increases Fos activation in the MEA and VMH following copulation.[435] However, methamphetamine treatment also makes female rats less selective in preference for particular males.[501]

Disinhibition may also occur as a function of hormone priming. Systems that normally maintain inhibition over female sexual behavior during nonovulatory periods likely must be inhibited to allow the behavior to occur. This appears to be the case for some systems in the VMH that help to time the behavior and that help to bring about estrus termination (see below). Thus the brain is set up with modules that gather and interpret sensory input and generate competent motor outputs at optimal times. This allows reward systems to be activated when females engage in the right behaviors at the right times, and allows such behavior to optimize the reproductive outcomes. The fact that paced copulation is both rewarding to females and results in stronger copulatory stimulation from males, leading to a greater chance of successful impregnation, is a good example of the intricate timing systems that blend the two. Far more is known about the neural and neurochemical control of lordosis than appetitive sexual approach, solicitation, or pacing behaviors. These are outlined below.

Sexual Approach Behaviors

Mesolimbic DA is involved in the sensitization and crystallization of incentive responding,[38] especially within terminals in the NAc. Microdialysis studies have shown that DA in the NAc increases during copulation in OVX female rats or hamsters primed fully with EB and progesterone. In rats the increase is approximately 150%

of baseline when females are presented with a gonadally intact, sexually vigorous male rat behind a screen, and increases to approximately 180% when the screen is removed and copulation ensues.[488] The temporal resolution of microdialysis (e.g., 10-min samples), however, does not permit specific behaviors to be correlated with the rise in DA especially during copulation. However, the degree of release is positively correlated with the number of attempts made by the female to nose-poke through the wire-mesh divider. DA release increases more in the NAc and dorsal striatum in hormonally-primed females that must make an operant response to gain access to males compared to females that do not,[487] suggesting that mesolimbic and striatal DA release helps orient the female toward a sexually active male and make approach responses in anticipation of sexual stimulation and reward.

Solicitations

The pioneering work of Wallen et al.,[57] McClintock,[50] and Erskine[49] refined Beach's[39] general category of "proceptive" behaviors in female macaques and rats into species-specific descriptions of sexual approach, solicitation, and pacing. In rats, solicitations could be defined as full (headwise orientation to the male followed by a runaway) or partial (hops and darts, and perhaps also ear-wiggles) depending on how close in proximity the female was to the male, and whether she had room to run away (e.g., Figure 50.5). Pacing could be operationalized as an intermount or inter-intromission interval imposed by the female, and could be assessed in open fields or as exits and returns to the male's side in a unilevel pacing chamber (e.g., Ref. 49), or level changes per mount or intromission in bilevel pacing chambers (e.g., Ref. 75). Female mounting of sexually sluggish or naïve males was considered by Beach[106] to be a "super-solicitation behavior" that would allow females to "show" the male what they wanted. Such behavior has been studied extensively in rats using sexually experienced OVX females primed with EB alone or EB + progesterone and given access to castrated male rats.[221,502–504]

Hoshina et al.[505] were the first to show that axon-sparing excitotoxic lesions of the mPOA abolished both full and partial solicitations, but enhanced lordosis, in sexually-experienced OVX rats primed with EB and progesterone. Lesions of the mPFC inhibited the temporal patterning of full solicitations, disrupting the runaway component.[506] In contrast, lesions of the lateral septum or VTA did not alter the frequency of hops and darts.[507,508] Lesions of the mPOA, VMH, or MEA, abolished the mounting of sexually sluggish males in OVX rats primed with EB + progesterone, whereas crystalline implants of estradiol to the VMH, but not the mPOA or MEA, induced the behavior in OVX rats.[221]

Role of Incertohypothalamic DA

As with lesions of the mPOA, systemic administration of DA antagonists such as haloperidol abolish solicitations but augment lordosis.[509,510] This effect does not appear to be mediated by mesolimbic DA, as 6-OHDA lesions of VTA DA neurons projecting to the NAc did not alter solicitations in small chambers.[511] However, DA in the mPOA plays a key role in the stimulation of solicitations, with D2 receptor activation facilitating solicitations in OVX rats treated with EB alone, and D1 receptor activation facilitating solicitations in OVX rats treated with EB and progesterone.[491,492] DA projections to the mPOA originate in the zona incerta (ZI). DA turnover in the ZI increases with estradiol administration.[512] Extracellular DOPAC, a DA metabolite, increases in the mPOA in the afternoon to the early evening of proestrus, around the time that sexual behavior is activated.[489] DA is also released within the mPOA in OVX rats treated with EB and progesterone, but not in rats treated with EB alone.[81,160,424,513] Infusions of SCH-23390, a D1 antagonist, to the mPOA of OVX rats primed with EB + progesterone significantly reduced solicitations selectively.[492]

Role of MCs

MCs like α-melanocyte stimulating hormone (α-MSH) are derived from POMC, a precursor peptide made largely in the ArcN from which is also derived the opioid β-endorphin and ACTH.[514] α-MSH synthesis is stimulated by estradiol[515] within ArcN neurons. Projections of those neurons terminate in the mPOA and secrete α-MSH. Two MC receptors exist in the brain, MC3 and MC4, of which the latter is found in the mPOA. Bremelanotide (formerly PT-141) is a synthetic analogue of α-MSH and is the active metabolite of melanotan-II (MT-II). Systemic administration of bremelanotide stimulates solicitations selectively in female rats primed with EB or EB + progesterone.[516] MT-II produces a weaker effect,[517] although five consecutive days of MT-II administration produces an effect similar to bremelanotide in magnitude.[518] The enhancement of solicitations by bremelanotide is duplicated by infusions to the lateral ventricles or mPOA, but not the VMH.[30] Systemic bremelanotide also stimulates DA release in the mPOA, but not NAc or dorsal striatum of OVX rats treated with EB and progesterone. Finally, the stimulation of solicitations by systemic bremelanotide can be reversed by infusions of a selective MC4 antagonist (HS019) to the mPOA, but not VMH.[30] It can also be reversed by infusions of the D1 antagonist SCH-23390 to the mPOA,[30] suggesting that incertohypothalamic DA terminals in the mPOA contain MC4 receptors that drive DA release which in turn stimulates solicitations by acting on D1 receptors in this brain region. Thus the integration between MC and DA systems in the mPOA is a critical component in the regulation of solicitations.

Glutamate in the Ventrolateral VMH

Full and partial solicitations are also under the control of the VMH. In a series of studies, Georgescu et al.[519,520] investigated the inhibitory role of glutamate neurons in the ventrolateral VMH on the sexual behavior of female rats. Both full and partial solicitations were inhibited dramatically by infusions of glutamate, and also by infusions of the selective ionotrophic receptor agonists AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainite to sexually experienced OVX rats primed with EB and progesterone.[519] Conversely, infusions of the dual AMPA/kainite receptor antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and DNQX (6,7-dinitroquinoxaline-2,3-dione) to the ventrolateral VMH of sexually experienced OVX rats primed with EB alone increased full and partial solicitations.[520]

Differential Effect of Opioid Receptors

Pfaus and Pfaff[521] reported that infusion of the delta opioid agonist DPDPE, but not the kappa opioid agonist U50-488h, or the mu opioid agonist DAMGO, to the lateral ventricles of sexually experienced OVX rats primed with EB alone or EB and a low dose of progesterone (to induce moderate lordosis and low solicitations) increased solicitations in bilevel chambers significantly over control infusions. It is not known at present where in the brain this facilitation of solicitations may occur.

Taken together, these data suggest that the connections between the VMH and mPOA are critical in the regulation of solicitations relative to lordosis. This is evident from the fact that mPOA lesions suppress solicitations but augment lordosis, and is an example of the kind of mutually-exclusive behavioral patterns discussed by Konorski.[522] Females cannot hold a lordosis posture while making a forward-directed solicitation and vice-versa. Mutually interactive inhibitory subsystems in the two regions likely regulate the timing of solicitations and lordosis.

Pacing Behavior

The ability of female rats to pace the copulatory contact is critical for the timing of intromissions. This timing leads to distributed stimulation of the clitoris, vagina, and possibly also the cervix, stimulation that female rats find rewarding,[81,160,513] and that facilitates pregnancy or the induction of pseudopregnancy,[49,523] and partner preference.[77,78] Female rats pace at a faster rate early in the copulatory interaction, but with successive ejaculations, the number of level changes per mount in bilevel chambers (Figure 50.5) increases dramatically, thereby increasing male inter-intromission intervals.[75] A similar effect is also observed in large open fields, and is more pronounced in wild rats compared to domesticated laboratory rats.[50] In unilevel pacing chambers, the latency to return to the male's side after mounts, intromissions, and ejaculations shows a progressive increase in time, which increases with successive ejaculatory series.[49,74] Low, steady rates of pacing are induced in OVX rats by EB and progesterone, whereas OVX rats administered EB alone show higher rates of pacing and rejection responses. Fully primed female rats tested in small chambers that offer no escape from the male also display rejection responses (e.g., rearing and boxing postures[524]) at a higher rate than females tested in chambers where they can escape. It would appear that females use rejection responses to pace the copulatory contact if they cannot do so otherwise. However, in those conditions, copulation does not induce reward and does not facilitate pregnancy or pseudopregnancy (see below).

Very little work has been done examining the neural control of pacing. However, Xiao, Kondo, and Sakuma[525] found that bilateral radiofrequency lesions of the lateral septum, but not the mPOA, disrupted the pattern of female exits from the male side of a 3-hole unilevel pacing chamber. In general, females with lateral septal lesions did not leave the male side after mounts, and took significantly more time than sham-lesioned females to leave the male side after intromissions or ejaculations. This suggests that activation of the lateral septum by copulatory stimulation is an important component of the regulation of pacing. It is not known whether such lesions would facilitate or inhibit the development of place or partner preferences. Guarraci, Megroz and Clark[526] found that cell body lesions of the mPOA, but not MEA or BNST, increased the intromission and ejaculation contact-return latencies of females in pacing chambers, and increased the number of withdrawals from the male's side following intromissions, suggesting that mPOA activation is critically involved in keeping females with males, consistent with its role in solicitations. Interestingly, clitoral anesthesia induced by lidocaine injections also increased the number of exits and returns displayed by OVX, EB and progesterone-primed rats in a 4-hold unilevel pacing chamber, decreased the amount of time spent with males, and increased the ejaculation return latency.[163] This indicates that CLS maintains low rates of pacing, which increase if the stimulation is blunted. As noted above, polysynaptic clitoral afferents project to the mPOA and CLS activates Fos in medial regions of the mPOA of female rats. It is not known if the reward induced by CLS is eliminated by mPOA lesions, although such lesions disrupt the reward induced by VCS[527] (see below).

Lordosis

More is known about lordosis than any other behaviorally relevant spinal reflex with supraspinal control, except perhaps the control of penile erection[172] and the conditioned eye-blink response.[528,529] The seminal work

of Pfaff[32,62] merged electrophysiology with anatomy and pharmacology and behavioral neuroendocrinology with molecular biology to determine the modular supraspinal components of the lordosis reflex and its control by ovarian hormones acting on receptors in the brain (Figure 50.19). The action of steroids on those receptors alters the brain's neurochemistry and activates the excitatory sexual systems reviewed above. This activation then alters the reaction of the female to incentive sexual stimuli, which leads her to being attracted to male cues. Similarly, it results in approaches and solicitation of sex from the male and, upon simple palpation of the flanks and perineum, the female no longer reacts with violent intense rejection but rather with ear wiggles and sexual receptivity. Thus, the behavioral reflex is linked by the mechanics of gene transcription and translation in critical hypothalamic circuits to the timing of ovulation so that the two can co-occur.

Coordinating physiological responses with behavior requires timing. Hormone priming essentially sets up a timing system for lordosis onset and offset. Although some of the neurochemical systems involved in onset are part of the excitatory system, others are actually inhibitory and keep lordosis from occurring too soon. Likewise, activation of the excitatory neurochemical systems keeps the potential for lordosis on long after the female has taken herself out of the mating game. In general, the hypothalamic targets of estradiol include neurosecretory neurons such as GnRH and DA neurons that affect both pituitary secretion and sexual behavior, and local circuitry neurons such as POMC, GABA, and glutamate.

Given the pivotal role that the VMH and the surrounding area plays in the control of lordosis behavior, our discussion will focus on this region, with the understanding that estradiol also affects the neurochemistry within the broader network of brain regions with ERs. Based on the global effects of estradiol and progesterone on VMH activity, as described below, ovarian hormones clearly transform neural processing in this brain region.

Lordosis Onset

Activation of Excitatory Systems

Estradiol and progesterone activate gene expression for a number of neurochemicals systems in the hypothalamus, most notably in the mPOA and VMH, which stimulate female sexual behaviors including lordosis (Figure 50.20). This includes an upregulation of specific neurotransmitter receptors by estradiol, e.g., progestin preceptors, OT receptors, adrenergic α_1 receptors, muscarinic receptors, MC three and four receptors, and delta opioid receptors, GnRH receptors, GABA-A receptors, and D1 DA receptors.[62,484] This changes how the circuit between the two regions operates. Enzymes are also upregulated in this system, including NO synthase, prostaglandin-D synthase, and DBH, leading to an upregulation of the end products. NO in particular is a critical and ubiquitous player in neurotransmitter release, so its upregulation is important in helping to set the stage for the upregulated neurochemical systems to play a functional role in the generation of the behavior. Pharmacological studies help to confirm the role played by these neurochemical substrates:

GABA AND GLUTAMATE

Throughout the central nervous system, many synapses include excitatory or inhibitory amino acids as neurotransmitters, namely glutamate and GABA, which bind to ionotropic receptors, allowing brisk changes in the postsynaptic potential. Both glutamate and GABA, and their ionotropic receptors, are present in the VMH.[531–538] Several methodological approaches have demonstrated ovarian hormone regulation of these neurotransmitter systems and implicated their actions in the VMH in the control of lordosis behavior (reviewed in Refs 536,539–541), providing insight into the neurological control of mating behavior.

Glutamate neurotransmission in the VMH has been studied with many technical approaches. Double-label immunohistochemical studies indicate that the ERα-expressing neurons in the VMH are glutamatergic, based on the colocalization of the vesicular glutamate transporter-2.[542] Ultrastructural analysis of the VMH has revealed abundant excitatory inputs, including axospinous synapses, which comprise approximately one third of the total VMH synapses. Based on hormone replacement regimens, estradiol treatment increases glutamate levels in the VMH, and this is reversed with subsequent progesterone treatment.[543] The number of AMPA and NMDA (N-methyl-D-aspartate) receptor subunits are likely to increase with estradiol treatment.[538,544] Although the behavioral pharmacological evidence is not complete, glutamate action in the VMH acutely inhibits female sexual behavior during the time window of receptivity.[541] This may be mediated by a combination of kainite, AMPA, and NMDA receptors.[519,520] Thus, it appears that female reproductive behavior depends on the inhibition of glutamate neurons in the VMH.

Whereas glutamatergic neurons in the VMH are the direct target of ovarian hormone action, the specific functions of GABAergic cells in the VMH are not yet clear. Nevertheless, GABAergic activity in the VMH is clearly modulated by estradiol. Estradiol treatment increases GABA levels in the VMH, based on hormone replacement regimens, and subsequent progesterone treatment returns GABA back to vehicle-treated levels.[545,546] Similarly, estradiol treatment may also increase GABA turnover.[546] The estradiol-induced increase in GABA production may be based on transcriptional regulation of GAD65, an enzyme involved in GABA biosynthesis,

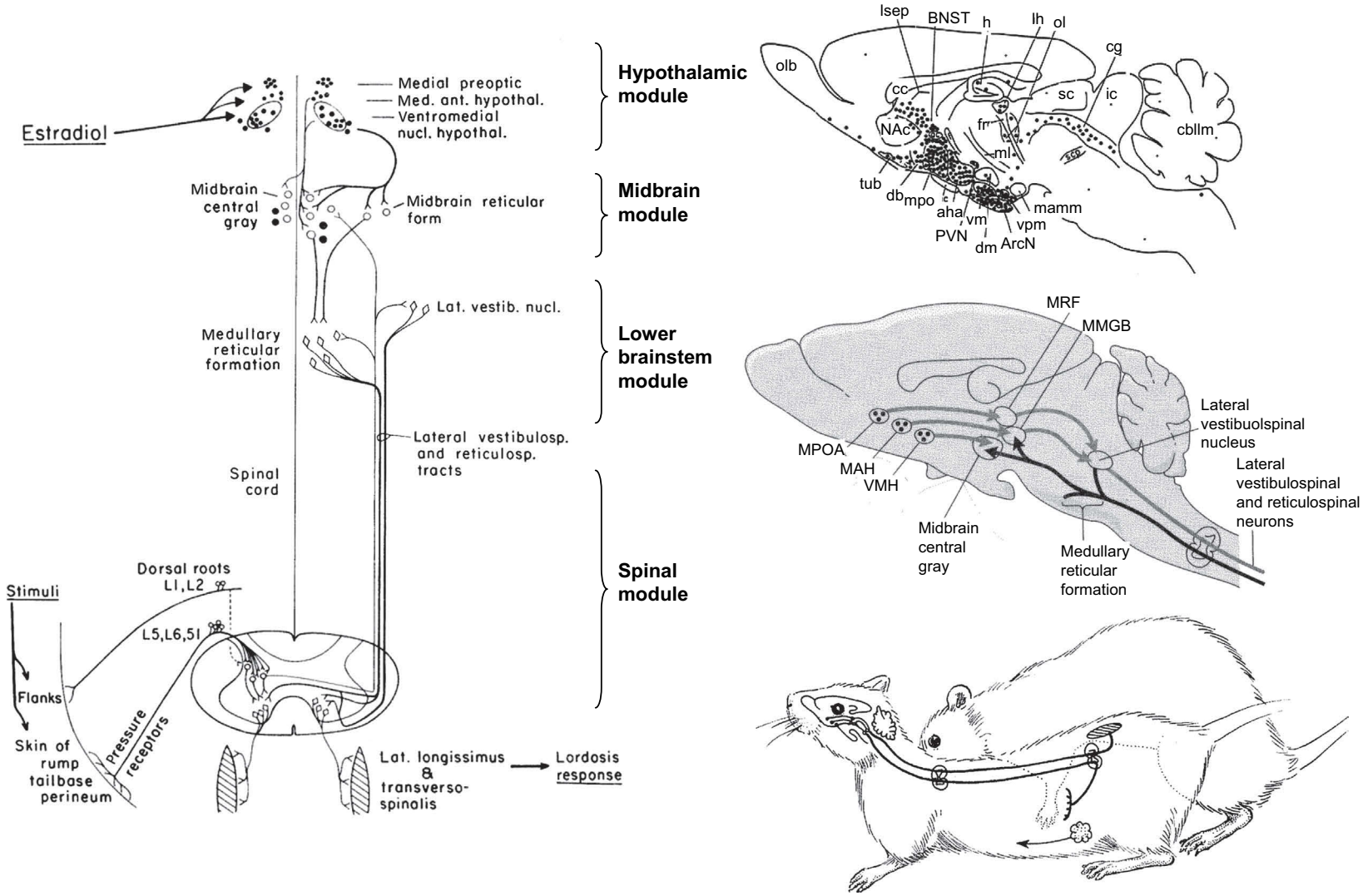

FIGURE 50.19 **Modular control of lordosis.** Left: Estradiol binds at the level of the hypothalamic and midbrain modules, altering the way that incoming somatosensory inputs from flank and perineal stimulation via the spinal cord to brainstem are processed and responded to. This alters the response of the female to palpation of the flanks, switching her from fighting to lordosis (bottom right). Right top: estradiol binding in the mediobasal hypothalamus, and midbrain of female rats. Right middle: critical elements of the lordosis circuit depicting regions of estradiol binding in the medial preoptic area (mPOA), ventromedial hypothalamus (VMH), and anterior hypothalamus. Motor pathways for the circuit include the lateral vestibular nucleus (for postural orientation) and lateral vestibulospinal and reticulospinal neurons that control contractions of the lateral longissimus and transversospinalis muscles of the back, which raises the base of the tail and head. Estradiol activates both nuclear and membrane-bound receptors in the hypothalamus to induce these changes, which include the augmentation of neurochemical systems that are excitatory for sexual behavior (see text). Abbreviations: aha, anterior hypothalamic area; ArcN, arcuate nucleus; BNST, bed nucleus of the stria terminalis; cblm, cerebellum; cc, corpus callosum; cg, midbrain periaqueductal gray; db, diagonal band of Broca; dm, dorsomedial hypothalamus; fr, fasciculus retroflexus; h, hippocampus; ic, inferior colliculus; lh, lateral habenula; lsep, lateral septum; MAH, medial anterior hypothalamus; mamm, mammillary bodies; MMGB, medial region of the medial geniculate body; mpo, medial preoptic area; ml, medial lemniscus; NAc, nucleus accumbens; ol, nucleus of the lateral olfactory tract; olb, olfactory bulb; PVN, paraventricular nucleus; tub, olfactory tubercle; vm, ventromedial hypothalamus; vpm, ventral premammillary nucleus. *Source: Modified from Pfaff.*[32]

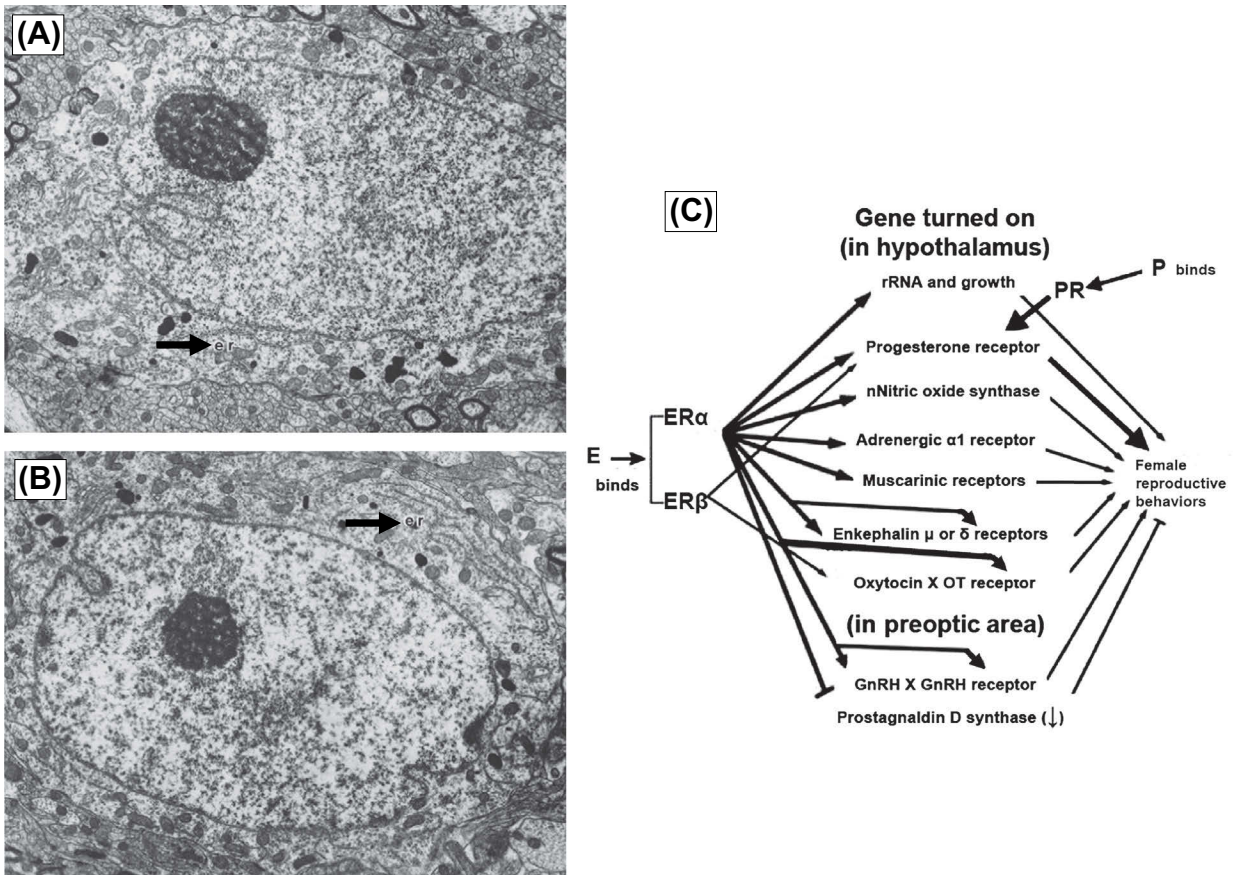

FIGURE 50.20 (A) Electron micrograph of a cell body in the VMH from an OVX rat treated with oil. (B) Electron micrograph of a cell body in the VMH from an OVX rat treated with estradiol (E) for 3 days. Note the accumulation of rough stacked endoplasmic reticulum (e r shown by arrow) indicative of protein synthesis in B compared to the smooth and unstacked, e r in A. *(Source: Adapted from Meisel and Pfaff.[448])* (C) Activation of excitatory neurotransmission in the lordosis circuit by E and progesterone (P) acting on ERs and PRs to modify gene expression. *Source: Adapted from Kow and Pfaff.[530]*

by ERα and ERβ.[547] Behavioral pharmacology experiments suggest that GABA activity may facilitate female sexual behavior,[543] thus exerting disinhibition.[539] GABA may work, in part, by inhibiting local serotonin release, which tonically inhibits lordosis behavior in the VMH,[543] and also by inhibiting glutamate neurons (see below).

These neurotransmitter systems also work in concert to exert opposite effects in the mPOA and VMH on lordosis. In the VMH, infusions of the GABA-A receptor agonist muscimol to OVX, EB-primed rats facilitates lordosis,[548,549] whereas infusions to the POA inhibit lordosis.[549] Infusions of muscimol also reduce extracellular 5-HT concentrations in the VMH.[548] Conversely, infusions of the GABA-A receptor antagonist bicuculline to the mediobasal hypothalamus resulted in an inhibition of lordosis. VMH infusions of antisense oligodeoxynucleotides that flank the start codon for two isoforms of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD; GAD65 and GAD67) inhibited lordosis in rats primed with continuous diffusion of crystalline estradiol in sc silastic capsule implants.[550] These data suggest that GABA in the mPOA inhibits lordosis by an action at GABA-A receptors, but facilitates lordosis in the VMH.

Glutamate was first reported by Kow et al.[540] to inhibit lordosis in OVX, EB-primed rats following infusions to the VMH. This effect was replicated and extended by Georgescu and Pfaus[519,520] who showed that infusions of glutamate or its ionotrophic receptor agonists AMPA, NMDA, and kainate, to OVX rats primed with EB and progesterone inhibited lordosis. NMDA and kainate infusions also increase the display of rejection responses. In contrast, infusions of the NMDA antagonist AP-5 or the AMPA/kainate antagonists, CNQX or DNQX, each facilitate lordosis in OVX rats primed with EB alone.[520] Interestingly, an electrophysiological depolarization signature due to glutamate release in VMH slices was shown by Booth, Weyman, and Jackson[551] to be inhibited by the same treatments. Moreover, pharmacological agents known to inhibit lordosis systemically, such as the 5-HT1A agonist 8-OH DPAT, the mu opioid agonist DAMGO, bicuculline, and CRF all potentiate the in vitro VMH glutamate EPSP (excitatory postsynaptic potential) signature.

The local VMH GABA and glutamate circuits are modulated, in part, by major afferents arriving from the hindbrain somatosensory-arousal systems providing serotonergic, noradrenergic, histaminergic and cholinergic projections to the area lateral to the VMH proper,[426,552–559] innervating the long primary dendrites that extend into the lateral fiber plexus. The brainstem nuclei of origin for these neurotransmitters all express estrogen receptors.[269,397,560,561] In summary, the key neurotransmitters of fast synaptic neurotransmission, glutamate and GABA, are regulated by estradiol within the VMH and have opposing effects on female sexual behavior.

DOPAMINE

Systemic administration of DA agonists can facilitate or inhibit lordosis behavior in OVX rats primed with EB and progesterone or EB alone. Paradoxically, systemic administration of a range of doses of DA antagonists also facilitates lordosis, although the behavioral signature of the effect is different. As mentioned above, whereas DA agonists produce a small increase in lordosis, but a large increase in solicitation behaviors, DA antagonists produce a large increase in lordosis quotients but abolish solicitations. Both the inhibitory and facilitatory effects of systemic DA agonists appear to act through D2 receptors in OVX, EB primed rats, but on D1 receptors in OVX rats primed with EB and progesterone. DA release in the VMH facilitates lordosis. Infusions of apomorphine or DA to either the mPOA or VMH facilitate lordosis behavior in OVX rats primed with low doses of estrone, whereas infusions of the DA receptor antagonists haloperidol or α-flupenthixol to these regions inhibit lordosis, but only in OVX rats made highly receptive with high doses of estrone. DA release is also increased in the VMH during copulation.[426] As discussed above, crosstalk between D1 receptors and steroid hormone receptors in the VMH appears to play a role in the facilitation of lordosis by a cellular process of ligand-independent activation of steroid hormone receptors. Mani et al.[413] reported that the D1 agonist SKF-38393 facilitates lordosis in OVX, EB-primed rats following infusions to the lateral ventricles, and that this effect is blocked by the progesterone receptor antagonist RU-38486 or by infusions of and antisense oligodeoxynucleotide directed against the start codon of the PR-A. These data indicate that activating D1 receptors in the VMH is capable of activating PR, possibly by altering the phosphorylation of the PR or a specific transcription coregulator. Thus, in addition to progesterone altering the activity of diencephalic DA systems, DA in the VMH appears to facilitate lordosis by the indirect activation of PR.

NOREPINEPHRINE

There is considerable evidence that the hormonal changes that underlie lordosis behavior and certain neuroendocrine reflexes, such as the preovulatory LH surge and pseudopregnancy, are associated with altered norepinephrine transmission. Early studies suggested that hindbrain noradrenergic projections to the hypothalamus provide critical visceral and somatosensory cues for female sexual behavior.[555] Extensive work by the Etgen laboratory has demonstrated that estradiol and progesterone enhance norepinephrine release in the vicinity of the VMH.[562] From a postsynaptic perspective, ovarian hormones reconfigure the populations of subtypes of noradrenergic receptors in the VMH, substantially altering signal transduction pathways and neurophysiological responses.[563] These changes in responsiveness and signal transduction channels appear critical for allowing the relevant sensory information to the VMH to promote the lordosis reflex.

Although systemic treatment with α or β noradrenergic receptor agonists and antagonists modulate lordosis, no clear picture emerges. Central effects of adrenergic drugs on female sexual behavior have not been studied in detail. Infusions of the α_1 antagonist prazocin into the VMH, but not the mPOA, inhibit lordosis, whereas infusions of the α_2 antagonist idazoxan or the β antagonist metoprolol to the VMH have only small inhibitory effects in some animals. Infusions of metoprolol to the mPOA inhibit lordosis in most rats. These results indicate that stimulation of α_1 receptors in the VMH facilitates lordosis, whereas stimulation of β receptors in the mPOA may inhibit lordosis. Consistent with this, in vivo microdialysis studies have shown that copulation with intromission increases extracellular norepinephrine concentrations in the VMH.[426]

ACH AND HISTAMINE

Both Ach and histamine have similarly been implicated as arousal neurotransmitters acting in the VMH to promote female sexual behavior, based on behavioral pharmacology studies.[564,565] As with serotonin and norepinephrine, specific subtypes of receptors for both of these signals are regulated by ovarian hormones.[566,567] Electrophysiological studies indicate that during estradiol exposure these signals have an enhanced excitatory effect on VMH neurons.[530,567] Histamine, in part, acts through H3 receptors to inhibit spontaneous GABA release.[532] The overall pattern that arousal transmitters that facilitate lordosis are also excitatory to VMH neurons would seem paradoxical, given that glutamate, a major excitatory transmitter, appears inhibitory for female sexual behavior within the VMH. In this regard, it is important to remember that the VMH includes several cell types that may be differentially regulated and exert excitatory vs inhibitory effects on behavior.[444] In addition, the effect of glutamate on neurotransmission may not be as straightforward as "excitatory". The net effect of glutamate on the membrane potential may be

dependent on the general state of excitability.[551] Nevertheless, there is a strikingly consistent pattern for hindbrain arousal pathways to converge on the VMH, with effects being modulated by estradiol.

OXYTOCIN

One of the most studied neuropeptides in female sexual behavior is OT, which acts in both the mPOA and VMH to promote female sexual behavior.[568,569] OT immunoreactivity is present in the lateral fiber complex, in axons arising from the PVN, colocalized with glutamate.[451] These OT axons innervate the long primary dendrites that extend into the lateral fiber complex.[451] Receptors for OT in the VMH undergo striking regulation by estradiol and progesterone in the VMH.[570] In general, ovarian hormones increase OT signaling by increasing levels of the receptor in the vicinity of the OT fibers. Electrophysiological studies suggest that OT has excitatory effects on VMH neurons.[571,572] Thus, OT may be co-released with glutamate in the area surrounding the VMH to signal social and sexual cues, and ovarian hormones sensitize specific VMH neurons to this information.

Within the VMH, estradiol stimulates OT receptor transcription. Lordosis is facilitated dramatically following systemic and intracerebroventricular administration of OT, and OT receptors in the mPOA and VMH appear to facilitate the frequency and duration of lordosis, respectively.[573] OT infusions to the VMH of OVX, estrogen-primed prarie voles reduces rates of aggression and increases the amount of physical contact that the females made with males, although it also leads to a faster termination of estrus. Copulation with intromission induces Fos within OT neurons of the paraventricular hypothalamus, but not the SON,[574] suggesting that endogenous OT systems are activated during copulation and may participate in the facilitation of lordosis following intromission. Interestingly, masturbation to orgasm increases plasma OT levels in women.[575]

GnRH, PROLACTIN, AND CHOLECYSTOKININ

Like OT, GnRH and prolactin have various effects that promote reproduction in females, including the promotion of female sexual behavior.[576–579] GnRH-labeled neurons reside in more anterior regions of the rat hypothalamus, but axonal fibers surround the VMH[580] and GnRH receptors are expressed in the VMH.[581] Likewise, prolactin receptors are expressed in the ventrolateral VMH,[582] and prolactin-immunoreactive axons are found in the mediobasal hypothalamus.[583]

GnRH produces a dramatic facilitation of lordosis in OVX, estradiol-primed rats following systemic or ventricular administration. Infusions of GnRH into the anterior POA, arcuate, and PAG, also facilitate lordosis in estradiol-primed rats, whereas infusions of a GnRH antibody into the MCG reduce lordosis quotients. Copulation with intromission, or manual VCS induces Fos within GnRH neurons of the anterior POA,[584] indicating that these neurons are activated during copulation. Interestingly, estradiol enhances the proportion of GnRH cells that express Fos following VCS, possibly through long-term noradrenergic activation. These results suggest that the activation of OT and GnRH neurons may form part of the system that facilitates lordosis following VCS. The behavioral effects of prolactin have not received as much experimental attention as GnRH, although there is a perception that sexual satiety is induced by prolactin in both men and women because prolactin release from the anterior pituitary into the general circulation occurs with orgasm.[585] Recently, it was found that women's self-reported satisfaction from orgasm is correlated positively and significantly with the concentration of postorgasmic prolactin found in serum.[586] Additional studies are needed to understand the neural circuitry, neurochemical coding, and neurophysiological targets of both GnRH and prolactin.

Another peptide that robustly innervates the VMH and mPOA is cholecystokinin (CCK). Immunoreactive terminals for CCK innervate VMH soma and dendrites, arising from the lateral parabrachial nucleus,[587–589] a region with strong ERα expression, and making symmetric synapses suggestive of inhibition.[590] Likewise, CCK receptors are found in the VMH.[591,592] However, the significance of CCK action in the VMH for lordosis and the type of information being relayed remains unclear.

DELTA OPIOIDS AND SUBSTANCE P

Two peptides intrinsic to VMH neurons are enkephalin and substance P. Enkephalin immunoreactivity is present in inhibitory axons terminating on GABA-containing VMH neurons. Thus, enkephalin may modulate these intrinsic GABAergic VMH neurons, thereby disinhibiting VMH output.[534] Enkephalin itself is upregulated by estradiol and progesterone in the VMH,[496] like its apparent co-transmitter GABA. Electrophysiological studies suggest that enkephalin has excitatory effects on VMH neurons. As with GABA, the delta opioid receptor agonist DPDPE inhibits or facilitates lordosis following infusions to the mPOA or VMH. Infusions of DPDPE to the lateral or third ventricles facilitates lordosis in OVX rats primed with EB alone or EB and progesterone.[521,593] A similar action was found when low doses of DPDPE were infused directly to the VMH.[593] However, higher doses inhibit lordosis. These effects were blocked by the delta opioid receptor antagonist naltrindole, and infusions of naltrindole alone to the VMH inhibited lordosis. Infusions of antisense oligodeoxynucleotides that flank the start codon for pre-proenkephalin mRNA to the VMH reduced lordosis quotients in OVX rats primed with estradiol.[594] As mentioned above, estradiol stimulates the synthesis of pre-proenkephalin in the VMH

which could act as an endogenous ligand for delta receptors there.[595] However, infusions of DPDPE to the lateral mPOA inhibit lordosis.[499]

Substance P is intrinsic to VMH neurons, and many substance P-labeled neurons express ER.[596,597] These substance P neurons are thought to project to the periaqueductal gray to promote sexual behavior.[598,599] Substance P-labeled terminals are likely to be excitatory, based on ultrastructure.[600] Thus, although both enkephalin and substance P are present in the VMH and promote lordosis, evidence suggests that they comprise distinct elements within the lordosis circuitry, with enkephalin modulating local inhibitory connections and substance P modulating the descending excitatory outflow.

In sum, the local VMH circuit includes glutamatergic and GABAergic neurons, which are directly and indirectly responsive to estradiol, and which have acute effects on lordosis behavior. The neuropeptides substance P and enkephalin are co-localized with these amino acids, and are likely to modulate their actions. The VMH also receives ascending influences from the hindbrain carrying arousal and sensory information, which is also modulated by ovarian hormones. Although the neurochemistry of many hypothalamic connections is not known, peptides associated with reproduction, such as OT, GnRH and prolactin may also affect lordosis by acting on VMH neurons. Taken together, the VMH is transformed by exposure to estradiol to change its internal pattern of activity, and at the same time, the salience of various inputs to the VMH is drastically recalibrated. As we better define the neurochemistry that controls the lordosis circuit, it will be important to understand how these neurotransmitters and neuromodulators work in concert to affect neurophysiology and behavior.

Inhibition of Inhibitory Systems

Tonic inhibitory systems exist for lordosis in the mPOA and VMH that must be overridden to activate lordosis. This action then constrains lordosis to the periovulatory period. Local actions of both mu and delta opioids, and glutamate, appear to keep lordosis constrained until such time as those systems are either inhibited directly, or the action of excitatory neurochemical systems overcomes the inhibitory tone.[35]

Serotonin is the most intensely studied transmitter in this category, and as might be expected, projections from the raphé to the VMH inhibit female sexual behavior.[601,602] Treatment of OVX rats with behaviorally effective doses of estradiol plus progesterone significantly reduces the turnover of serotonin in the VMH in a manner that correlates with changes in lordosis behavior.[603] Decreased serotonin in parallel with increased sexual behavior is also seen across the estrous cycle.[489] This inhibitory effect of serotonin on female sexual behavior is associated with an inhibitory effect on VMH neuronal activity, although the affected cell types have not been described. The serotonin receptor subtype 2C has been localized to the VMH and its neuropil.[533] Acute systemic treatment with the selective serotonin reuptake inhibitor fluoxetine disrupts estrous cyclicity, and reduces lordosis and the amount of time OVX rats primed with EB and progesterone spend with males, with Sprague-Dawley rats being less sensitive to these effects than Fischer rats.[604–606] The reduction in lordosis by fluoxetine was attenuated by progesterone,[607] and by chronic daily exposure to males.[606]

Timing Mechanisms

There are at least two timing mechanisms that have been explored, one that keeps the onset of sexual behavior in females tightly linked to ovulation, and another that allows females to receive a requisite amount of genitosensory stimulation before they fall out of heat. Sinchak et al.[608] have carefully provided evidence for a hypothalamic timing microcircuit in which estradiol acting at ERα in the ArcN stimulates neuropeptide Y release locally within the arcuate that in turn stimulates the activation of POMC neurons that project to the mPOA. These neurons release β-endorphin which activates mu opioid receptors in the mPOA, internalizing them and effectively inhibiting lordosis for the duration of its action. The maintenance of the mu opioid receptor internalization is provided by the activation of GABA-B receptors in the arcuate. This mechanism would also be expected to inhibit appetitive solicitations derived from activation of DA in the mPOA. However, this transient inhibition is itself inhibited by subsequent progesterone actions at PRs, and/or by higher doses of free estradiol acting at membrane receptors in the ArcN (linked to Gq-coupled activation of Srx) and/or in the mPOA (linked to GPER-30 receptors) to deactivate mu opioid receptors in the same membrane.[609,610] Together, these mechanisms create a biochemical timer that keeps sexual behavior from occurring until requisite physiological changes have taken place to support pregnancy. It may also be an important mechanism to investigate for estradiol-induced negative feedback, which may augment opioid actions in the mPOA while reducing the GPER-30 linked disinhibition.

Lordosis must also be maintained long enough for females to receive sufficient CLS for reward and potentially cervical stimulation to induce the neuroendocrine reflexes (e.g., upsuck for sperm transport, increased prolactin surges) that facilitate pregnancy and the progestational state. Glutamate neurons and glutamate transmission in the VMH are activated by VCS and inhibit lordosis as part of a local hypothalamic circuit that terminates estrus (see below). Estradiol augments GABA turnover in the VMH, and glutamate neurons

have GABA-A receptors, making them a target of inhibition by GABA. Such a mechanism likely extends the period of sexual receptivity before glutamate release in the VMH reaches a critical threshold for estrus termination.

It is tantalizing to consider that estradiol tips a balance between energy regulation and sexual behavior by modulating hypothalamic systems that promote feeding, utilizing them to inhibit both appetitive solicitations and lordosis until ovulation, at which time mechanisms that normally inhibit feeding (e.g., MCs) are activated that stimulate sexual behavior (e.g., Refs 611,612). For example, infusions of antisense oligodeoxynucleotides to GAD, which stimulate lordosis, inhibit food intake in rats.[613] This contrasts dramatically with the ability of food and sex to activate the central nucleus of the amygdala and stimulate mesolimbic DA release similarly in the NAc.[614,615] This suggests a fundamental difference in the way that hypothalamic regulatory systems and more general mesolimbic incentive motivational systems regulate different motivational states. Hypothalamic systems appear to regulate different motivations as if each possesses its own separate and mutually exclusive drive state. In contrast, the mesolimbic incentive system treats external stimuli associated with those drive states as essentially similar in the induction of forward-directed locomotion. However, it is also the case that glutamate in the VMH inhibits both feeding[616] and sex,[519,520,540] and may be involved in satiety-related mechanisms that terminate both motivational systems.

Estrus Termination

The offset of appetitive sexual responses and lordosis occurs as the period of sexual receptivity terminates. This is typically referred to as "estrus termination". Part of this process accompanies the natural decline in hormonal titers, whereas more immediate inhibition stems from an abbreviation of the sexually appetitive and receptive period brought about by VCS from intromissions and ejaculations.[617] Abbreviated estrus induced by mating stimulation or VCS has been shown to occur in rats,[75,617,618] guinea pigs,[619] and hamsters.[620] Indeed, allowing females to pace sexual contacts increases the inhibitory effect of intromissive stimulation on estrus duration in estrus-cycling rats[74,523] presumably because males make deeper and more powerful thrusts that could stimulate the cervix. In fact, OVX rats primed with EB and progesterone and given 50 distributed VCSs with a glass rod (that approximates the number of intromissions they would receive in an hour from a male) display no solicitations, low levels of lordosis, and high numbers of rejection responses when tested 12 h later.[75] In that study, rats given sham stimulation 12 h before testing displayed normal rates of solicitations and lordosis, and no rejection responses. Females allowed to copulate freely with males in bilevel chambers show changes in the intensity of appetitive and consummatory behaviors, such that by the fourth ejaculatory series, fully primed female rats show few solicitations, lower lordosis frequencies and reflex magnitudes, longer pacing intervals, and higher numbers of rejection responses, compared to the first ejaculatory series.[70] It is around this time that naturally-cycling female rats in large semi-natural environments will take themselves out of the open area and back into the burrow system, effectively ending their participation in sexual activity. However, in experimental settings where sexual stimulation is not controlled adequately by the female, she displays longer periods of sexual receptivity than would be predicted from studies in the wild or in semi-natural environments. This is similar to the observation by Wallen et al.[621] of female rhesus macaques displaying nearly constant receptivity when paired in small enclosures with a single male, relative to females in large natal groups that take themselves out of a sexual interaction by retreating to the female territory.

Role of PRs

Inhibition of protein synthesis by infusion of a protein synthesis inhibitor into the mPOA in hamsters[622] or following systemic administration to guinea pigs[619] blocks mating-induced abbreviation of the period of sexual receptivity. Likewise, inhibition of protein synthesis delays heat termination.[623] Recent work suggests that heat termination that is hastened by mating stimulation is referable to more rapid down-regulation of PRs (specifically PR-B)[354] than the down-regulation in response to progesterone. Thus, although there is still much that is not known about the role of PR-A and PR-B in particular neurons, it is clear that PRs play a key role in the timing of sexual receptivity in a variety of circumstances by serving as a gatekeeper for the transcriptional processes within those neurons involved in sexual behavior.

Although, there is a temporal correlation between decreased blood levels of progesterone and termination of behavioral estrus,[362] the two events are not causally related. That is to say, the period of sexual receptivity ends even when levels of progesterone are maintained.[624,625] Because of the importance of PRs to the facilitation and maintenance of sexual behavior, the cellular basis for heat termination requires looking at the regulation of PRs. Progesterone down-regulates its own receptors. Loss of behavioral response can typically be attributed to either a declining concentration of activated/occupied hypothalamic PRs[356] or the absence of a sufficient level of progesterone to interact with the particular level of unoccupied receptors. The decline in concentration of unoccupied PRs can come about in a variety of ways; a decrease in estradiol levels results in the loss of induction of PRs, and exposure to

progesterone down-regulates PRs. Both processes typically occur in tandem.

It has been suggested[356,626] that the refractory period, which follows termination of sexual receptivity[236,627,628] comes about as a result of the same mechanism that causes heat termination—down-regulation of PRs by progesterone. During the refractory period, the concentration of hypothalamic PRs is depressed in relevant brain areas,[357,358,629,630] and progesterone treatment results in low levels of activated PRs.[362,631] In addition, a supplemental estradiol injection, which offsets the decrease in the concentration of unoccupied PRs, resulting in high levels of occupied PRs in response to progesterone,[364] causes the animals to regain response to a second progesterone injection.[236,628,632] The refractory period can also be overcome by injection of a large dose of progesterone,[631,633] which, unlike a lower dose, results in a large increase in progesterone-occupied PRs in the hypothalamus.[631] Therefore, with a variety of conditions, there is a strong concordance between the level of activated PRs and the expression of lordosis.

There have been conflicting reports of progesterone involvement in estrus termination in rats. However, although rats may not become completely insensitive to progesterone after estrus termination, they do in fact become hyposensitive to it[634] The hypothesis that estrus termination and the refractory period are both due to loss of activated PRs may explain the conflicting opinions concerning progesterone's role in estrus termination.[224,635,636] Perhaps behavioral response is critically dependent on an adequate concentration of PRs, rather than progesterone per se.

Role of Disinhibited VMH Glutamate

As mentioned above, VCS that induces estrus termination activates a population of glutamate neurons in the ventrolateral VMH[637] (Figure 50.21). When OVX rats are given 0, 1, 5, 10, 20, 30, 40, or 50 distributed VCSs, the activation of Fos in those neurons is delayed by prior treatment with EB or EB and progesterone, relative to the oil-treated control, such that fewer neurons reach a threshold for activation until animals receive between 10 and 20 VCSs.[169] This blunted activation indicates that steroid hormones have suppressed the ability of VCS to activate these inhibitory glutamate neurons, although by 50 VCSs there is no difference in the number activated between steroid-treated and control groups. How might estradiol with or without progesterone do this?

Virtually all the glutamate neurons that co-express Fos after VCS also contain GABA-A receptors which are up-regulated by EB treatment.[638] The stimulation of GABA synthesis by EB in GABA neurons that project to the VMH would be expected to bind to GABA-A receptors on the inhibitory glutamate neurons and induce IPSPs for an extended period of time, long enough perhaps for the female to receive enough intromissions and ejaculations to ensure pregnancy, or at least the progestational state consonant with pseudopregnancy. Extracellular concentrations of glutamate in the VMH during copulation in OVX females are very high in females injected only with oil (which reject male advances), and lowest in females primed with EB and progesterone who display full appetitive and consummatory sexual responses.[638] Indeed, infusions of the AMPA/kainate receptor antagonist DNQX to the VMH prior to the application of 50 distributed VCSs delayed the induction of estrus termination observed in saline-treated controls 12 h later.[639] The source of the GABA input to glutamate neurons has not been identified, although preliminary findings suggest it comes largely from the mPOA.[640] Thus blocking glutamate receptors in the ventrolateral VMH blocks the abbreviation of estrus induced by VCS, an effect that may occur naturally by estradiol-induced inhibition of glutamate neurons. The net effect would be for females to remain sexually receptive long enough to receive a requisite number of intromissions and ejaculations to ensure pregnancy.

Disorders of Sexual Desire or Interest in Women

An important example of how basic research translates into clinical treatments comes from the study of the neurochemistry of sexual desire. At least three potential treatments for disorders of sexual desire or interest in women are being considered, including the MC agonist bremelanotide, the serotonergic mixed 5-HT1A agonist/5-HT2A antagonist flibanserin, and a combined pill containing testosterone and a phosphodiesterase-5 (PDE-5) inhibitor called Lybrido®. Acute bremelanotide increases solicitations selectively in preclinical models using OVX rats primed with low doses of EB, or low EB and progesterone.[30,516] Likewise chronic flibanserin increases solicitations and reduces rejection responses in OVX rats primed with EB or EB and progesterone.[316] Microdialysis samples from the mPFC, NAc, and mPOA showed that acute flibanserin increased basal levels of NE in all areas, along with DA in the mPFC and mPOA, but not the NAc. Acute flibanserin also decreased serotonin levels in all areas. However, chronic flibanserin increased DA and NE significantly in the mPFC, but did not alter serotonin, glutamate, or GABA relative to chronically injected controls. Finally, acute treatment of OVX rats primed with low EB with testosterone and a PDE-5 inhibitor increased solicitations and hops and darts.[641] All three drugs have shown significant efficacy in increasing self-reported sexual desire in pre- and post-menopausal women diagnosed with hypoactive desire disorder.[30,642–646] The ability of these three drugs to stimulate solicitations in a rat model of hypoactive sexual desire predicts their functional application in women with hypoactive sexual desire. This suggests strongly that the neurochemical systems underlying

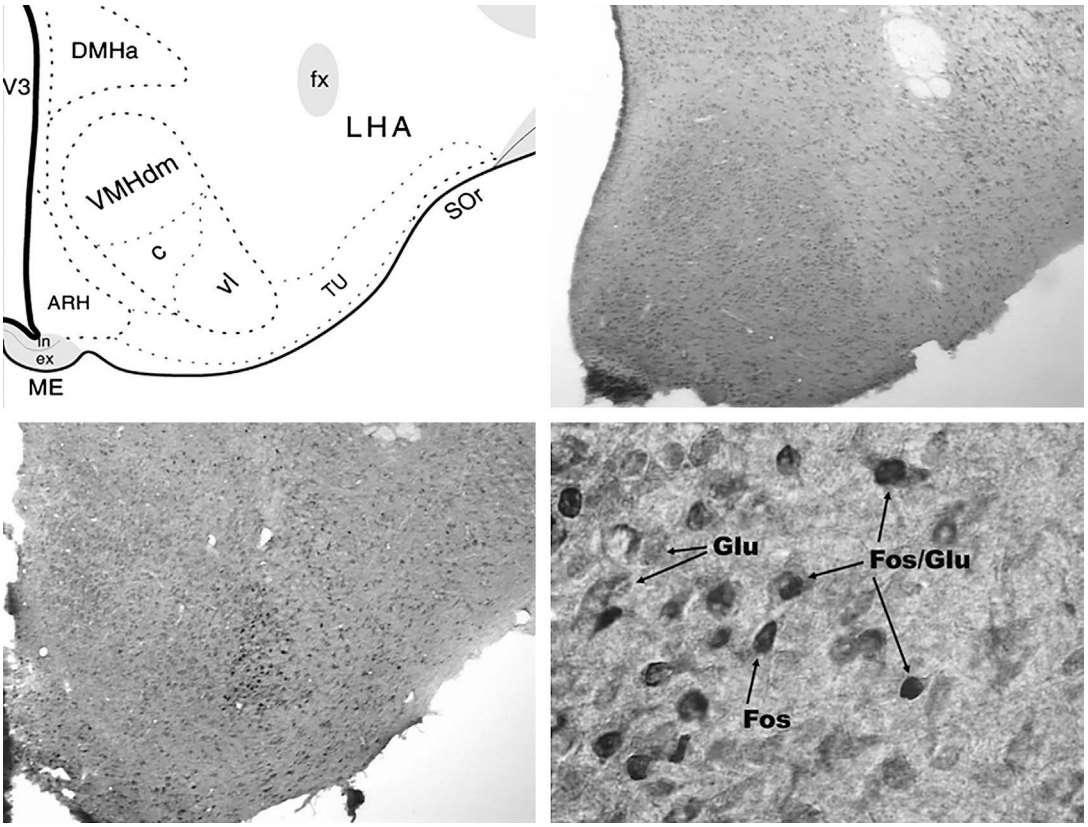

FIGURE 50.21 Activation of glutamate neurons in the VMH by 50 artificial vaginocervical stimulations over the course of 1 h. Top left: schematic drawing of the VMH and its substructures. Top right: glutamate staining in the VMH. Bottom left: Fos induction within glutamate neurons in the ventrolateral VMH. Gray cytoplasmic staining is for glutamate (Glu) whereas black nuclear staining is for Fos. Bottom right: close up of double labeling of Fos within Glu neurons, Fos alone (non-Glu neurons), and Glu neurons without Fos. *Source: Reprinted from Georgescu et al.,*[637] *with permission of Elsevier.*

appetitive sexual behavior are conserved between at least rats and humans, and that translational work on the neural and hormonal systems that mediate sexual responses in women can be derived from basic and clinical research in other species.

CONSEQUENCES OF SEXUAL STIMULATION

In addition to estrus termination and the activation of neuroendocrine reflexes associated with pregnancy and pseudopregnancy, stimulation of the clitoris and possibly also vagina and cervix induce a state of pleasure or reward. As with many rewarding stimuli, a neurobiological "preference" can be established for physical elements associated with the original pleasure-inducing event. For things such as pleasure-inducing drugs this can include the place in which the drug effect is experienced. In animal models this can be manipulated so that an individual's preference for one distinctive environment in which the drug effect was experienced, vs a different environment in which the effect of vehicle or placebo was experienced, can be quantified and used as an indicator of how rewarding the drug is.[647] This same principle can be applied to sexual stimulation received in one distinctive environment vs no stimulation in another environment. In both cases the location in which the rewarding event occurred becomes a conditioned stimulus in the classic sense and thus this phenomenon (or behavioral assay) is referred to as conditioned place preference (CPP). Specifically in the context of sexual reward experienced with a distinctive partner, the same principle can be applied such that salient partner-related cues become conditioned stimuli that induce a partner preference. Research into the role of conditioning as a result of sexual stimulation brings together three important observations in females (and males) of a variety of species.

First is the phenomenon of mate choice, observed naturally in female prairie voles that display monogamous social and sexual partner preferences with the first male they mate with (see Chapter 48). This phenomenon traces its cause in large part to the particular way that OT is activated in regions of the limbic system and hypothalamus that create an incentive sexual preference for a particular

male with whom the female has mated with.[648,649] The male of this species also displays monogamous social and sexual preferences,[650,651] and the two will form a partnership around care and raising the young. It has been assumed that most other species are polygamous, and in particular, sexually promiscuous. If there are preferences at all (especially in males) it is for a different partner every time in order to spread the genes far and wide in the pool. Both male and female rodents are assumed to be polygamous and promiscuous, with both showing "Coolidge effects" in which sexual activity is more vigorous with new compared to familiar partners.[72,652]

The second phenomenon is sexually CPP, as referred to above. In these studies rats or hamsters are placed into the start chamber of a CPP box (Figure 50.22) that contains two distinctive environments to either side that vary by floor pattern or some other distinctive characteristic but one that will not, in and of itself, invoke a preference. The sequential pairing of sexual stimulation or a reward state induced by sexual interaction with a partner is then experienced in one side, and no stimulation is experienced in the other side, and occurs for several trials after which the rat is placed into the start chamber and allowed to roam freely between the two distinctive environments. If the sexual stimulation has positive reinforcing (i.e., rewarding) properties then the rat will spend significantly more time on the side of the chamber where it previously experienced the rewarding sexual stimulation (UCS (unconditioned stimulus)).

Seminal work by Paredes and colleagues starting in the mid-1990s asked if female rats "liked" sex. Prior to this work, lordosis and other sexual behaviors were seen as being "driven" by estradiol and progesterone, but given the existence of penile spines it was far from clear that female rats found sexual interaction with males rewarding. And given the anesthetic properties of progesterone, it was indeed possible that they did not, but that the potential pain of sex was merely reduced to endurance levels by hormone action. In fact, in some

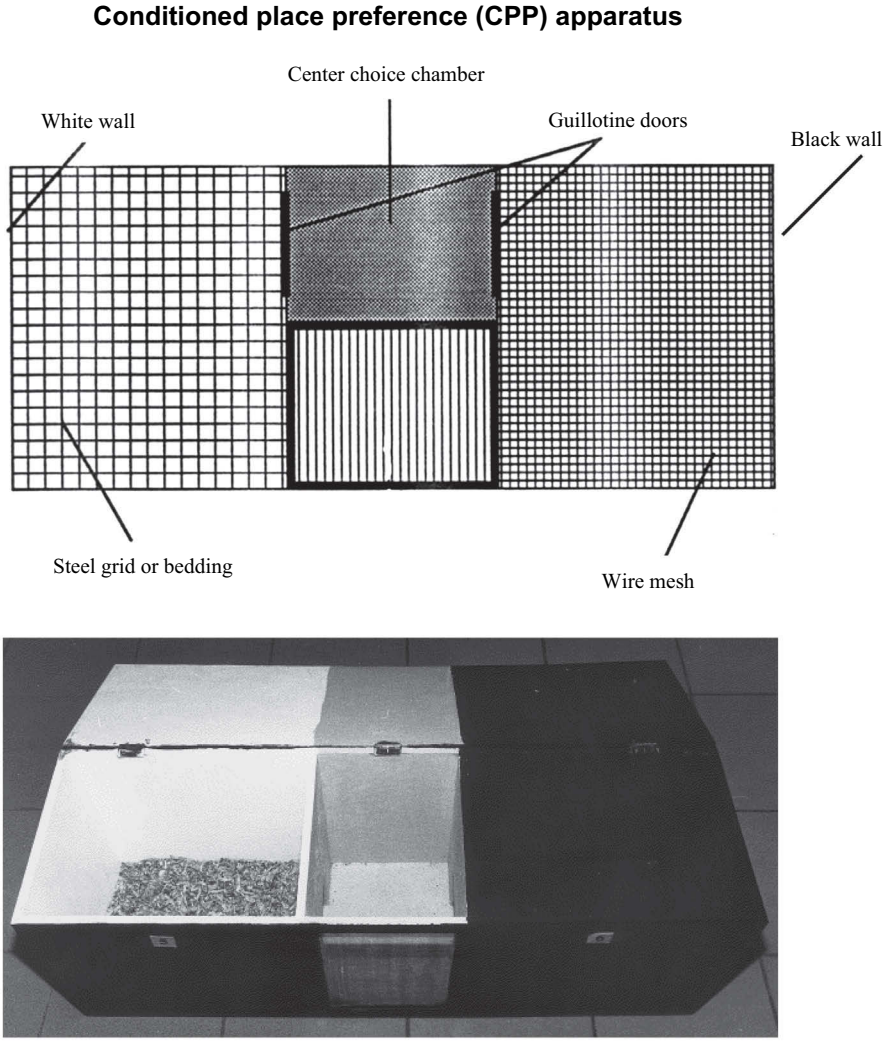

FIGURE 50.22 Typical conditioned place preference (CPP) apparatus used to determine the rewarding properties of drugs or sexual stimulation. *Source: Adapted from Paredes and Vazquez.*[81]

studies prior to Paredes' work, female rats were observed to run away from the male after ejaculation, or to choose to spend more time with a castrated male relative to an intact male.[653] Two important papers by Paredes' group changed that perception (see below).[81,513]

The third phenomenon merged the first two, showing that polygamous and promiscuous female rats are capable of developing sexually conditioned partner and mate preferences based on the degree of reward experienced during their first sexual encounters, and whether this sexual reward had been paired with a discrete odor cue (e.g., almond) on the male they had their first rewarding sexual experience with.[28,78] This effect was also demonstrated for the strain of the male with which the female had her first sexual experience.[77] Female rats also display mate guarding behavior, just as female prairie voles do, if they are sexually receptive and placed into a situation with their male and another competitor female who is sexually receptive. Together, these phenomena link reward and reproduction, and bring them to a more cognitive level of analysis that translates extremely well to human sexual behavior. One significant advantage to the use of neutral stimuli such as an odor of a place being paired with a sexual reward state is that the stimuli can be presented alone—as a priming cue—and the activation of brain areas in response to the conditioning cue in the absence of the actual rewarding stimulus can be identified. This has led to important insights into the neurological underpinnings of reward, both in response to pleasure inducing drugs and sexual stimulation.[63]

Sexual experience in females also changes cellular morphology in critical regions of the reward circuitry. In female golden hamsters, for example, copulatory experience increases dendritic spine density in the NAc, but decreases it in the PFC[94] (Figure 50.23). An augmentation of synapses in the NAc, concomitant with a decrease in the PFC, would be expected to enhance the ability of distal sexual incentives to focus a female's attention and activate excitatory appetitive sexual responses. Thus, the ability of sexual experience to augment the activation of sexual reward, and in turn sexual desire, is most likely rooted in molecular, structural, and neurochemical changes that sensitize females to competent sexual incentives and cues that predict sexual reward or pleasure.

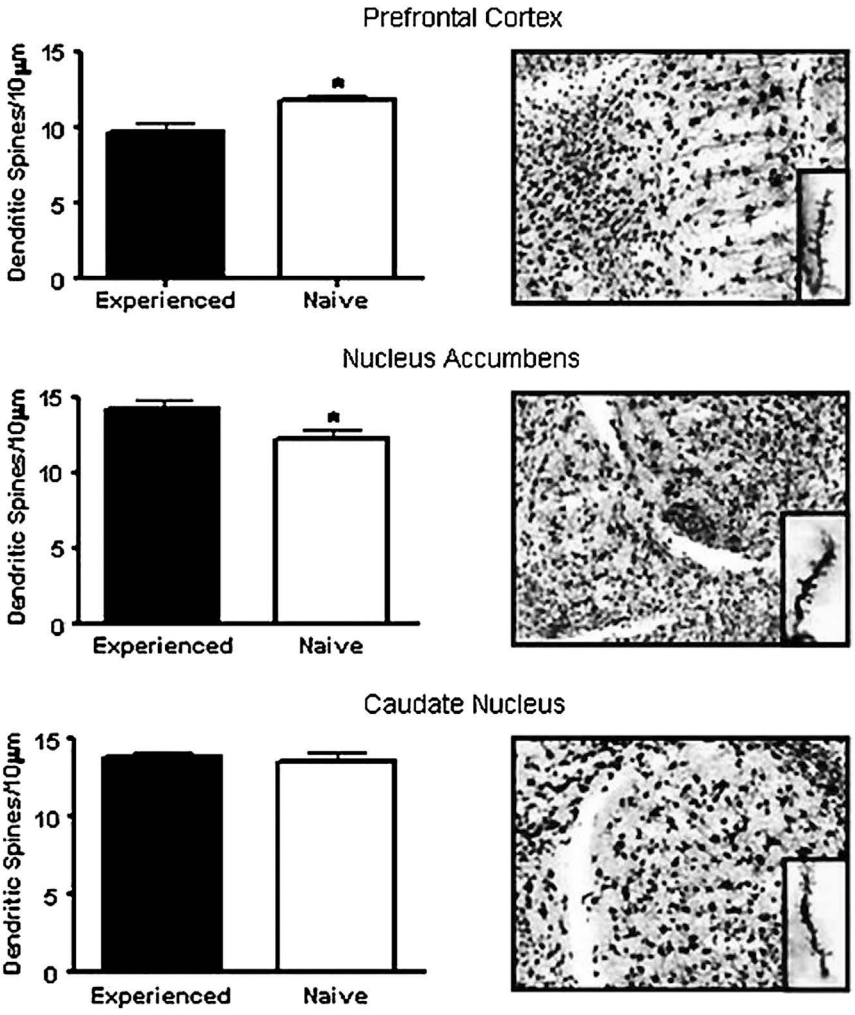

FIGURE 50.23 Spine densities of Golgi-stained neurons from the prefrontal cortex (PFC), NAc, or caudate nucleus (dorsal striatum) in sexually experienced or naïve female hamsters. Sexual experience induces a significant reduction in spine density in the PFC, but a significant increase in the NAc. This pattern of synaptic alteration would be expected to enhance reactivity to both unconditioned and conditioned sexual incentives. *Source: Reprinted from Meisel and Mullins,[94] with permission of Elsevier.*

Sexual CPPs in Females

Oldenburger et al.[654] found that when copulation occurred within one of the distinctive compartments of a CPP apparatus, female rats showed a weak preference for the chamber in which mating occurred. Subsequently, Paredes and Alonso[513] and Paredes and Vazquez[81] demonstrated a robust place preference in female rats when they were able to pace the rate of copulation without having to employ defensive behaviors. This was accomplished using unilevel pacing chambers bisected by a Plexiglas divider with one or more small holes that only the female can pass through.[74,81,513,523] The male was sequestered on one side of the chamber and the female was then free to pace the copulatory contact by running from side to side. Like males, females acquired a strong preference for a distinctive environment only if they were placed into the CPP box after paced copulation. No preference was found if the copulation was unpaced prior to placement in the CPP box (meaning that it had occurred in the same pacing chamber but without the divider). Thus, for a female rat, place preference develops only if she has been able to control the initiation and rate of copulation freely without having to use defensive behaviors.

There is an alternative interpretation of the place preference induced by controlled paced mating in females. Rather than paced mating being rewarding per se, it may be that under conditions in which the female has little control over the interaction, such as is usually the case in tests of animal mating behavior performed in small constrained arenas such as glass aquaria, that these interactions are actually aversive. To examine this possibility, Afonso, Woehrling, and Pfaus[504] allowed female rats to copulate in two unilevel pacing conditions using Plexiglas dividers that had either four holes or one hole. This was done to eliminate the possibility of an "aversive" state resulting from unpaced copulation. Trials were conducted sequentially at 4-day intervals and each pacing condition was paired with one of the distinctive sides of a CPP apparatus, in a counterbalanced fashion. Control groups contrasted the 4-hole or 1-hole condition with a no-divider condition (as was done by Paredes and Alonso[513]). Control females developed significant CPP for either the 1-hole or 4-hole condition, relative to unpaced copulation with no divider. These data replicate the findings of Paredes and Alonso[513] and indicate that both the 4-hole and 1-hole condition were rewarding relative to the unpaced (no divider) condition. However, they do not rule out the possibility that the real distinction being made was between an aversive condition (unpaced copulation) and a rewarding condition (paced copulation). This was addressed in the group allowed to contrast the 4-hole vs 1-hole condition, in other words females that could control the pace of the sexual interaction freely (in the 4-hole chamber, females can move freely from side-to-side) vs those that could not (in the 1-hole chamber, males often obstruct the hole with their heads, forcing the females to wait longer to get to the male's side). Females developed significant CPP for the 4-hole relative to the 1-hole paced copulation experience. Thus free control over the rate of copulation appears to be a crucial variable in the rewarding aspects of pacing. Similarly, Jenkins and Becker[655] found that female rats developed significant CPP for paced relative to unpaced mating, but also for unpaced mating in which the experimenter removed the male for a period that approximated the female's imposed interintromission interval, relative to unpaced mating in which male removal did not occur. Thus, female rats develop CPP for sex at their own preferred intervals. Taken together with the results of Matthews et al.,[656] these data suggest that reward comes from the sexual stimulation that females receive, namely mounts with intromission, so long as that stimulation occurs at the desired time intervals.

What is it about paced copulation that leads to CPP in females? Meerts and Clark[657] reported that VCS applied with a 1 ml syringe plunger at 200 g of pressure for 2 s at 30-s intervals, for a total of 15 stimulations, induces a reliable CPP in OVX females primed with estradiol and progesterone. Given that VCS could stimulate the internal clitoris as well as the cervix, we asked whether external CLS could induce CPP.[160,658] As mentioned above, in these studies CLS was administered either with a lubricated paintbrush or a small cotton-tipped vibrator at preferred intervals for 10–15 min over five to six reinforced sessions. Both types of stimulation induce robust CPP. Importantly, reward as a consequence of CLS can be induced in OVX females with or without hormone priming,[162] indicating that sexual reward is independent of steroid priming, although such priming would normally be required for females to experience CLS from mounts with pelvic thrusting, as it would be necessary to induce lordosis which exposes the clitoris to the male's perineum during pelvic thrusts.[164] Indeed, females primed sequentially with EB and progesterone, or its ring A-reduced metabolites, show enhanced CPP from paced copulation relative to females primed with EB alone, presumably due to the greater degree and frequency of lordosis induced in females receiving progesterone in addition to estradiol.[659] OVX rats primed with low doses of EB that induce low to moderate lordosis do not develop CPP,[660] OVX, hormone-primed rats given exitotoxic lesions of the nucleus paragigantocellularis of the brainstem have attenuated lordosis and appetitive behaviors.[661] Interestingly these females also do not develop CPP to artificially-applied VCS.

Maintenance of the memory of paced sexual reward does not require exposure to hormones. Parada et al.[162] attempted to extinguish paced copulation-induced CPP

by exposing OVX rats in three priming conditions (oil, EB alone, and EB+progesterone) to the CPP box without prior copulation. Only females primed fully with EB+progesterone shifted their preference back to the original preconditioning side. At first glance, this would appear to be counterintuitive, given that the reward state induced by distributed CLS (and possibly also paced copulation) is not hormone dependent. However, *desire* is hormone-dependent, and only the females that had hormone-induced activation of appetitive sexual motivation, but did not receive sexual stimulation, showed extinction of the CPP. Nonprimed, or EB-primed, females did not extinguish the CPP. This makes sense from a conditioning viewpoint: animals that are not in a state of desire do not need that state satisfied, whereas animals in a state of desire seek satisfaction of that desire. Extinction thus occurs only when a state of desire or need exists and there is no satisfaction.

Finally, the CPP induced by paced copulation in females can be blocked by systemic injections of naloxone,[662] or following infusions of naloxone to the mPOA, VMH, or MEA, but not the NAc.[663] Similar data have been reported for males, suggesting that common opioid systems in the brains of male and female rats are activated by sex-related cues[662] and constitute a primary reward signal. Bilateral lesions of the nucleus paragigantocellularis in the brainstem decrease the amount of time females spend with males, which in turn, attenuates the CPP induced by paced copulation.[621] Such lesions also attenuate the CPP induced by artificial VCS, suggesting both a behavioral and sensory deficit induced by the loss of the nucleus paragigantocellularis.

Conditioned Sexual Partner Preferences

As outlined above, female rats also show olfactory conditioned partner preference for males associated with a pacing-induced reward state.[78] This was accomplished in unilevel pacing chambers in which the paced condition involved the placement of either a 1-hole or 4-hole Plexiglas divider through which the female could regulate the initiation and rate of copulation. The nonpaced condition involved copulation in the same chamber but without the divider. Females in the paired group were given paced copulation with males that had almond odor applied to their necks and anogenital area vs nonpaced copulation with males that had distilled water applied to the same areas. After four paced vs nonpaced trials, females were placed into a large open field with two tethered males, one scented and the other unscented, and choice of male to solicit, copulate with, and receive ejaculations from, was recorded. Females for which the odor was paired with the paced condition selectively solicited, copulated with, and received ejaculations from the scented male. Females that had the odor explicitly unpaired or paired randomly with pacing did not display a preference (Figure 50.24).

As with males, females showed a similar preference for strain cues associated with paced copulation,[77] although it was stronger if the strain associated with paced copulation was their own. Interestingly, in that study, pigmented or albino females solicited whichever strain of male was associated with paced copulation, but received ejaculations preferentially from males of their own strain and only if that male had been associated with paced copulation. This also revealed a degree of assortative choice, especially for mating, and showed that females, like males, can differentiate copulation from mating. Finally, female rats that experienced manual distributed CLS in the presence of a cotton gauze pad soaked in almond extract chose to copulate selectively with almond-scented males over unscented males during their first sexual experience in a large open field with both males.[161] Interestingly, they did not show a preference to receive the scented male's ejaculations, suggesting that the VCS received from males during paced copulation induced a further reproductive or mate choice. It is not yet known whether this stems from specific stimulation of the cervix (and pelvic nerve) or from full stimulation of internal and external aspects of the clitoris,[166] or other sensory regions inside the vagina. Experience with paced, relative to nonpaced, copulation in unilevel chambers induces significant neurogenesis in the granular layer of the accessory olfactory bulbs,[664] a region known to contain intrinsic memory systems related to pheromonal stimulation and recognition of conspecifics.[665,666]

Female rats also learn inhibitory associations. Coria-Avila et al.[83] found that administration of the opioid receptor antagonist naloxone blocks the development of sexually conditioned partner preference in OVX female rats primed with estradiol and progesterone. Subsequent analysis of the naloxone training sessions revealed that by the 6th or 7th trial, most females display significantly fewer, if any, solicitations, a low frequency and magnitude of lordosis, and a far higher number of rejection responses, as if they were in a state of estrus termination (Figure 50.25). In some females this occurred before any intromissions were achieved by the males, although such stimulation was often reacted to violently by the females who would box and push the males onto their sides or backs. *And this was despite full hormonal priming with estradiol and progesterone.*

In addition to the inhibitory effect of sex without opioid reward induced by naloxone, thwarted sexual activity in the presence of an inaccessible male can also induce an inhibitory state. Parada et al.[161] gave sexually naïve female rats five trials of CLS in the presence of a sexually active male scented with almond behind a screen. On alternating days, the females received sham CLS in the presence of an unscented male behind the screen. During

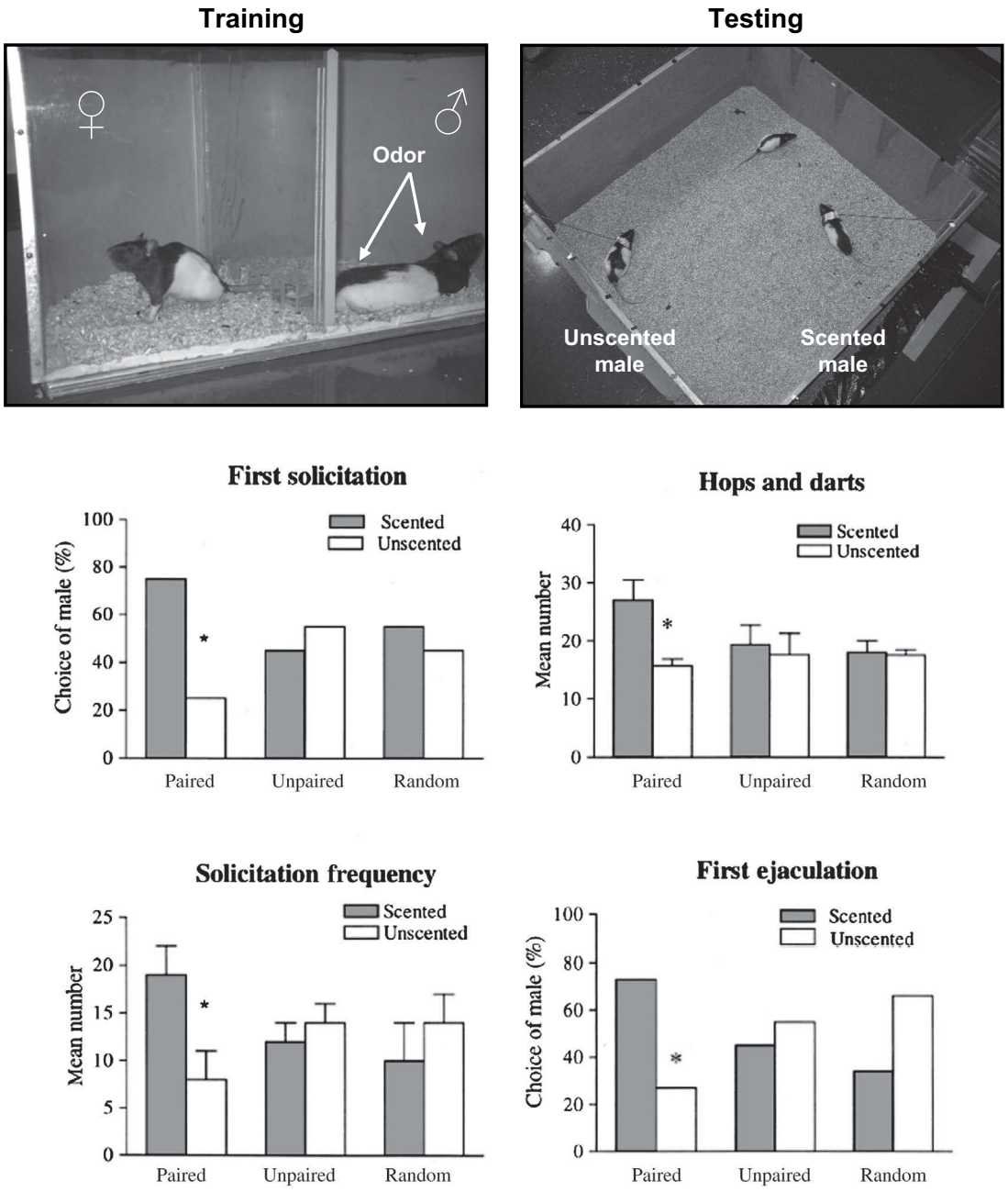

FIGURE 50.24 Conditioned partner preference in the female rat. Top: Paired females are given their first sexual experiences in a unilevel pacing chamber with a male behind a 1- or 4-hole divider scented with a neutral odor (e.g., almond). This is followed 4 days later by exposure to an unscented male but with the divider removed. After several sequential exposures of scent and paced copulation and no scent and unpaced copulation, females are tested in a large open field with two tethered males, one scented and one unscented. The choice of male for solicitations, hops and darts, mounts, intromissions, and ejaculations is recorded. Unpaired females are given the opposite order of association during training, no scent with paced copulation and scent with unpaced copulation. Random paired females are given scented and unscented males randomly associated with paced and unpaced copulation. Bottom: Choice of male for first solicitation, frequency of solicitations, hops and darts, and choice of male for first ejaculation, from paired, unpaired, or random-paired groups. *Source: Adapted from Coria-Avila et al.*[78]

the final open field test with two males, one scented and the other unscented, females solicited selectively the *unscented male* and showed a trend to receive that male's ejaculations preferentially. At first glance, these data seem at odds with the fact that CLS induces a reward state. However, it was noted that females attempted to solicit the males behind the screen following CLS during the training trials, which, of course, were not successful because the male was behind a screen. Thus, it is likely that the female was in a state of thwarted sexual nonreward that she associated with the odor and generalized to the choice of male for her first sexual experience.

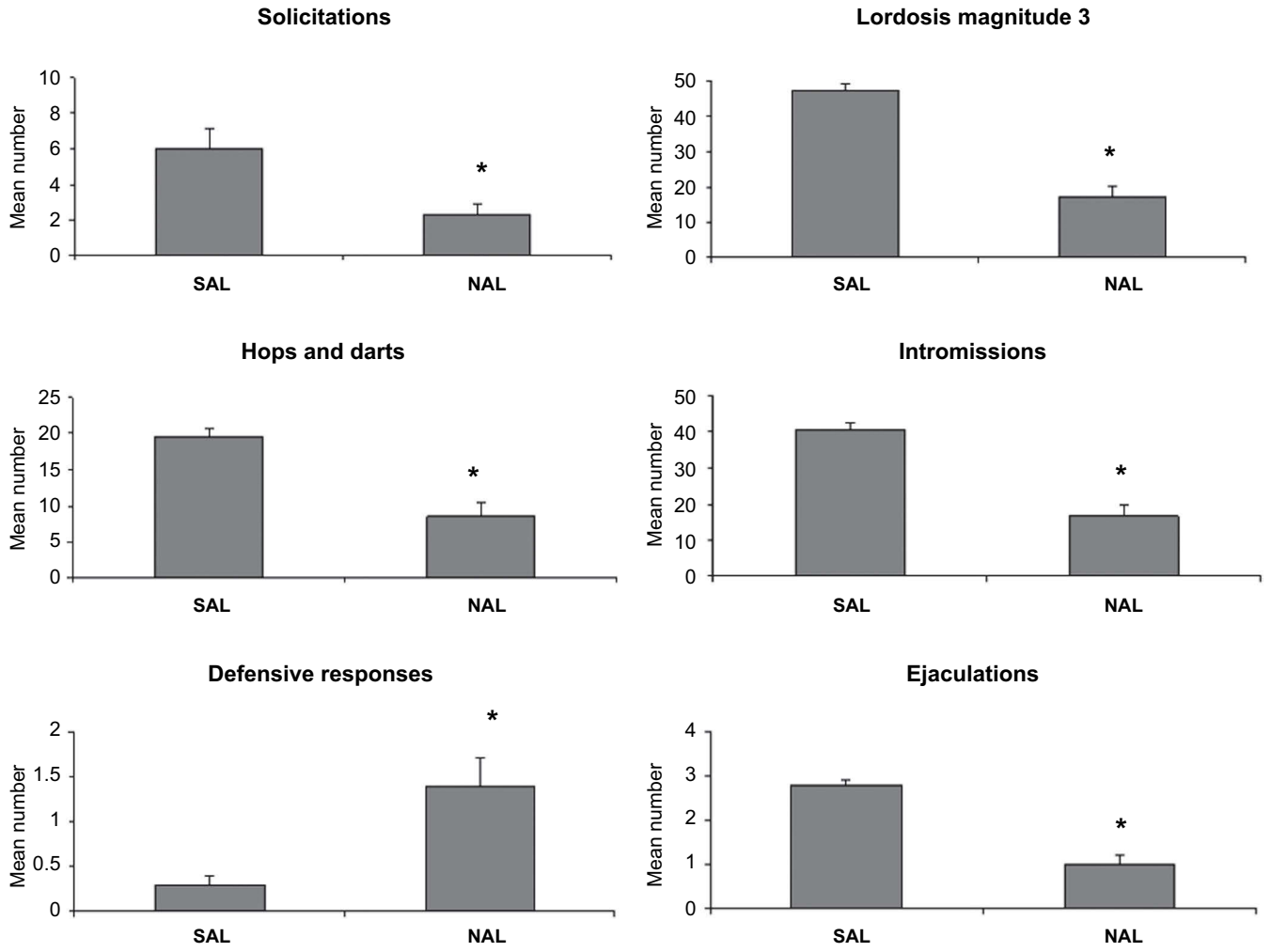

FIGURE 50.25 Effects of acquiring sexual experience under the influence of saline or naloxone (5 mg/kg, ip) on appetitive and consummatory sexual behaviors in OVX female rats primed fully with estradiol and progesterone. Females received six multiejaculatory experiences at 4-day intervals prior to the final test in which all rats received an injection of saline. *Source: Adapted from Pfaus et al.*[28]

After conditioning, the odor cue can be presented alone to examine its priming effect in the brain. Relative to the unpaired group, the odor presented to females in the paired group activates Fos (Figure 50.26) in a circuit that is strikingly similar to that observed in fMRI brain scans of women during the presentation of erotic pictures, and especially pictures of their partners.[64] These regions include: olfactory tubercle, piriform cortex, ACC, insula, NAc, dorsal striatum, lateral septum, mPOA, PVN of the hypothalamus, ArcN, VMH (scattered), and VTA.[76] Presentation of strain cues behind a wire-mesh screen also activated Fos. Relative to unpaired rats, Fos protein was activated in significantly more cells in the piriform cortex, mPOA, VMH, and VTA.[76] Areas of common activation by odor and strain related cues associated with paced copulation are shown in Figure 50.27.

Finally, OVX, hormone-primed female rats given their first 10 multiejaculatory sexual experiences with the same unscented male display agonistic behavior toward an OVX, hormone-primed competitor female (CF) when the three are placed into an open field. Sexually receptive female rats have generally been observed to compete with one another in large mating arenas[50]; however this behavior is reminiscent of the mate guarding displayed by Prairie voles.[667] The partner female (GF) typically mounts the competitor female (HO) and pushes her into corners of the open field, sometimes with aggressive postures, prior to running back to the partner male with her ears wiggling. Occasionally she positions herself between the male and the competitor, making it nearly impossible for the male to gain access to the other female.[668] These observations have been replicated and extended,[669] and brain activation by the encounter compared between the GF and the HO. In both cases, Fos activation of CLS and VCS zones in the hypothalamus and limbic system have been detected; however in GFs that position themselves more frequently between the male and the HO, Fos protein was induced in a greater number of cells by the copulatory stimulation.

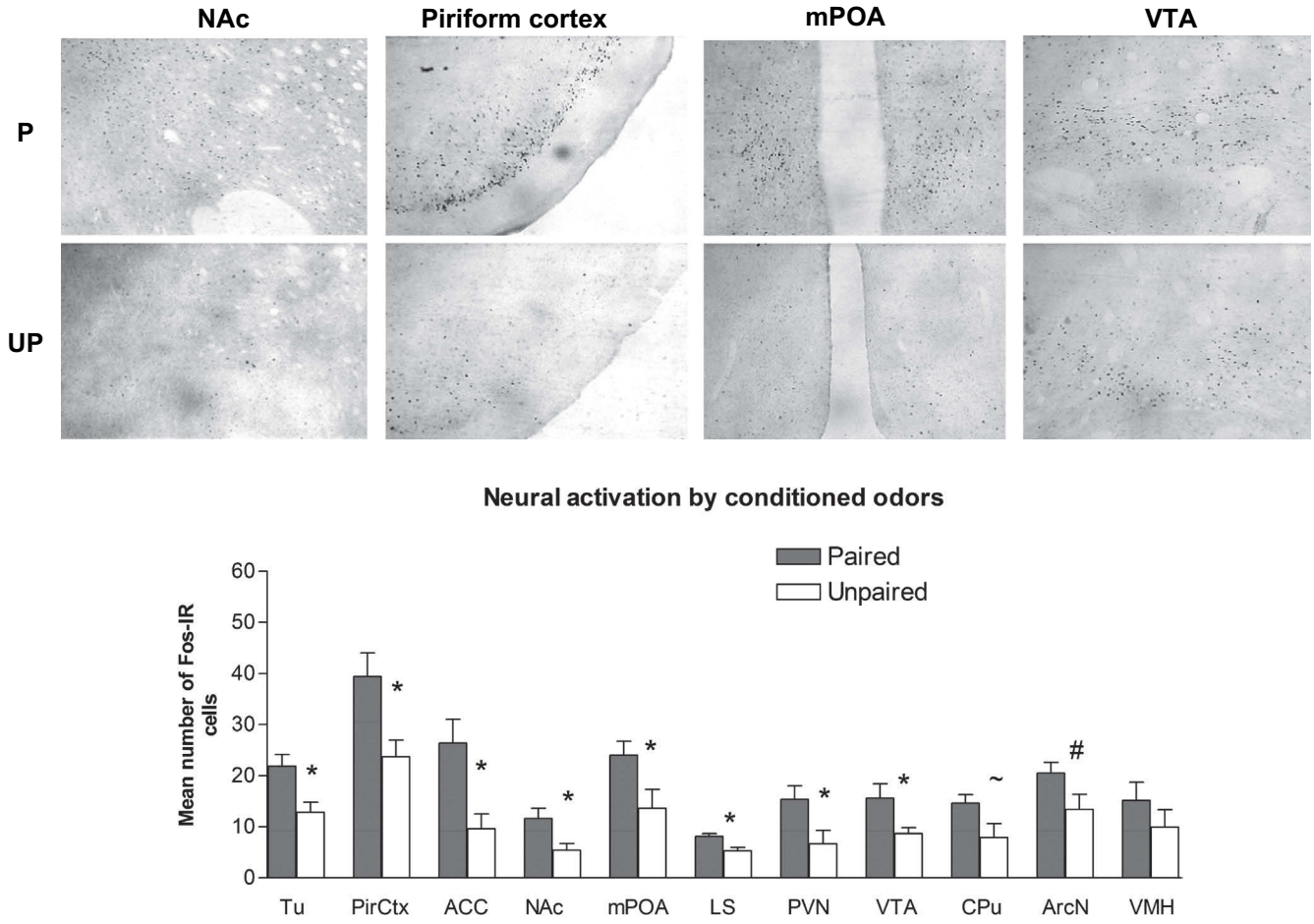

FIGURE 50.26 Selective activation of Fos by an almond odor paired (P) or unpaired (UP) with paced copulation. Note the activation of the central DA cell body-rich region of the VTA by the odor cue. Abbreviations: Tu, olfactory tubercle; PirCtx, piriform cortex; ACC, anterior cingulate cortex; NAc, nucleus accumbens; mPOA, medial preoptic area; LS, lateral septum; PVN, paraventricular nucleus; VTA, ventral tegmental area; CPu, caudate-putamen; ArcN, arcuate nucleus, VMH, ventromedial hypothalamus. * $p<0.05$; # $p = 0–.06$; ~ $p = 0.08$. *Source: Reprinted from Coria-Avila and Pfaus,[76] with permission of Elsevier.*

However, only the GF shows significant activation of Fos within OT and vasopressin neurons of the paraventricular and supraoptic nuclei, and within regions of the hippocampus and corticomedial amygdala that could indicate an additional stress response.

It would appear that female rats possess the ability to show behavioral and neuronal rudiments of either selective or promiscuous mating depending on their early sexual experience. Such experience seems to crystallize sexual response patterns and preferred sexual stimuli by sensitizing a circuit similar to that activated in monogamous prairie voles during their formative sexual experiences[96,651,670] and following parturition.[671] The results of Aragona et al.[96] are particularly instructive, as the sexual bond formation was inhibited by activation of D1 receptors, but facilitated by the activation of D2 receptors. This suggests a neural reorganization in mesolimbic terminals after formative sexual experiences that "seals the bond", making it less likely for other stimuli to acquire associative strength.

Such an effect is consistent with modern theories of learning (e.g., Refs 672,673) and has been implicated in the susceptibility to drug addiction, especially in terms of responding to cues that predict drug reward,[674] and more generally in response to food-related cues.[614,675] The interaction of OT and DA in the PVN, mPOA, VTA, and NAc of male rats induces penile erections and links them to appropriate appetitive sexual behaviors.[676] Thus, opioid reward states may form the rudimentary mechanism of bonding because they sensitize DA release in the presence of reward-related cues compelling animals to focus their attention and goal-directed behavior toward those cues. Activation of brain OT systems (by DA or other means) adds a reduced social distance and bonding to this neurochemical reward state. Given that pharmacological activation of opioid receptors induces a direct suppression of both hypothalamic and pituitary OT secretion,[677] sensitized and potentially reorganized mesolimbic and hypothalamic DA systems must be a necessary intermediary. This is consistent with a

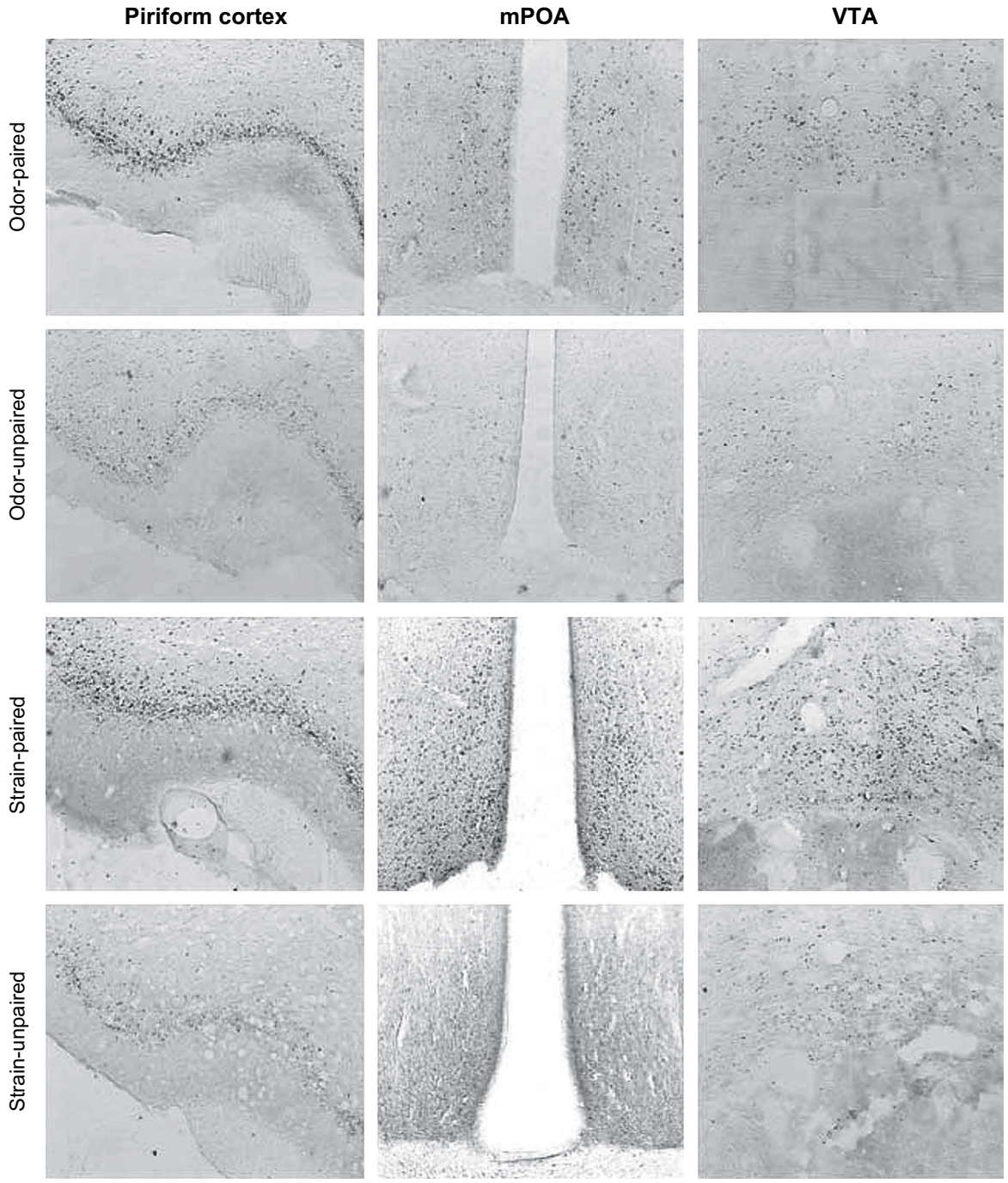

FIGURE 50.27 Comparison of Fos in the piriform cortex, mPOA, or VTA, by almond odor or strain cues (pigmented vs albino) paired with paced copulation. *Source: Reprinted from Coria-Avila and Pfaus,[76] with permission of Elsevier.*

multifaceted role of mesolimbic DA in incentive salience and response initiation.[38]

Conditioned Sexual Arousal in Women

It is difficult to condition sexual arousal or desire in adult humans as they have likely already had their sexual preferences set by experience long before. This is especially true in women when using erotic films or pictures as the UCS that are rated as mildly or moderately stimulating (e.g., Ref. 678). More recent attempts have been more successful using UCSs or CSs of higher incentive quality. For example, Both et al.[679,680] found that neutral pictures of male headshots paired with 2 s of intensely pleasurable vibrotactile CLS produced greater vaginal pulse amplitude (VPA) during extinction in the

paired vs unpaired groups. In another study, stimuli were presented briefly (30 ms).[681] Although only the paired group showed increased VPA to the CS during the first extinction trial, there was no increase in the conscious affective value of the stimulus. Finally, Hoffmann, Janssen, and Turner[682] varied the duration and relevance of a CS (abdominal area vs a gun) that was paired with short erotic film clips in both men and women. Interestingly, when the stimuli were presented subliminally for brief durations, the relevant abdominal stimulus increased arousal in both men and women. However, when the stimuli were presented for longer periods prior to the erotic film clips, a sex difference emerged in which the relevant CS alone (abdominal area) induced genital arousal in men, but the presumably irrelevant stimulus alone (gun) induced genital arousal in women. This latter effect may indicate that women require CSs that increase autonomic arousal to a higher extent than men, a potential corollary of the "discordance" experienced by women, but not men, between genital and subjective sexual arousal.[151] It may also be the case that anything that activates the sympathetic nervous system sufficiently in women may generate a genital blood flow response, regardless of whether the stimulus is sexual in nature. This may occur at anytime during the menstrual cycle, but may be interpreted as more "sexual" when women are periovulatory.

An Integrative Model of Conditioned Sexual Responding

The model proposed by Pfaus, Ismail, and Coria-Avila[683] and shown in Figure 50.28 accounts for both unconditioned and conditioned sexual responding, integrating a number of important regions that regulate female sexual responses by the hypothalamus, limbic system, and cortex. With reference to conditioning, CSs associated with sexual reward state UCSs act as "priming

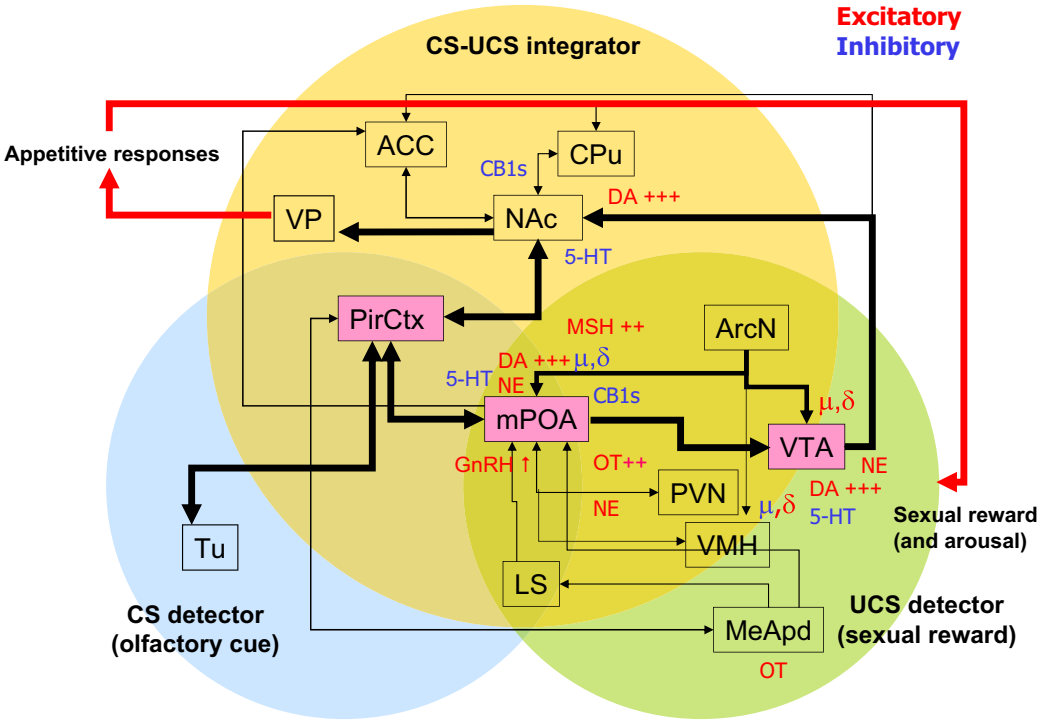

FIGURE 50.28 Neural systems critical for the display of sexual behavior in the rat. This figure is reproduced in color in the color plate section. Appetitive behaviors made toward unconditioned or conditioned sexual incentive stimuli lead to sexual reward that is processed by three interactive systems. Two systems process olfactory stimuli and sexual reward relatively independently, whereas a third, mesolimbic DA system, acts to integrate both the conditioned olfactory cue and its rewarding sexual outcome. Three common regions, the piriform cortex, mPOA, and VTA, are activated in male and female rats by conditioned olfactory stimuli. Opioid actions in the VTA potentiate mesolimbic DA activation, whereas opioid actions in the mPOA inhibit sexual arousal and desire. Neurotransmitter systems or their receptors in red are excitatory for sexual motivation whereas those in blue are inhibitory. Black pathways signify major inputs and outputs of the system. Note that opioids can be excitatory in the VTA, inhibitory in the mPOA, or either in the VMH (depending on the receptor type). A similar system is activated in humans.[63] Abbreviations: ACC, anterior cingulate cortex; ArcN, arcuate nucleus of the hypothalamus; CB1, cannabinoid Type 1 receptor; CPu, caudate-putamen (striatum); DA, dopamine; δ, delta opioid receptors; GnRH, gonadotropin releasing hormone; LS, lateral septum; MeApd, posterior-dorsal nucleus of the medial amygdala; mPOA, medial preoptic area; MSH, melanocyte stimulating hormone; μ, mu opioid receptors; NAc, nucleus accumbens; NE, norepinephrine OT, oxytocin; PirCtx, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; Tu, olfactory tubercle; VMH, ventromedial nucleus of the hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area; 5-HT, serotonin. *Source: Adapted from Pfaus et al.*[683]

stimuli" to activate cortical, limbic, and hypothalamic circuits involved in the facilitation of sexual arousal and desire and/or in the suppression of inhibitory systems (Figure 50.28). Some of those circuits involve selective processing of the sexual stimulation that generates the reward state (UCS), the olfactory stimulus (CS), and a system that integrates the CS and UCS so that animals can focus their attention and engage appropriate forward-directed locomotion toward the CS when it is present.[38,46] What becomes apparent is that systems for sexual reward and incentive responding overlap with systems proposed for sexual (and maternal) bonding (e.g., Ref. 670). These involve the interaction of at least three neurochemical systems, including mesolimbic DA, hypothalamic OT, and opioids that inhibit hypothalamic structures like the mPOA, but sensitize mesolimbic DA systems through a process of disinhibition.[684] This is strikingly similar to Fisher's[685] proposal of three primary emotional systems for mating, reproduction, and parenting, to the mechanism proposed for romantic love in humans,[686] and to the models of human sexual responding proposed by Georgiadis and colleagues.[45,64]

During conditioning, female rats display solicitations indicative of their desire to copulate. If they receive the appropriate stimulation (i.e., distributed CLS during paced copulation) UCS-detector centers become activated (green circle). The posterior-dorsal nucleus of the medial amygdala is sensitive to paced copulation and projects information about it to the mPOA which serves as a main integrator area. The VTA is activated to provide DA to areas important for motivation and decision making (e.g., NAc, ACC) and motor activity (caudate-putamen, ventral pallidum). POMC cells in the ArcN project to the VTA and release opioids that hyperpolarize inhibitory GABAergic neurons (not shown), and therefore increase DA cell firing in mesolimbic and mesocortical terminal regions. The ArcN also projects α-MSH and opioid terminals to the mPOA to facilitate solicitations and reward, respectively. OT terminals from the PVN may release OT into the mPOA to facilitate reward and bonding. Concurrently the Tu (blue circle) and PirCtx sense the CS. PirCtx projects olfactory information to the mPOA and to the NAc to strengthen the incentive value of the CS. Exposure to the conditioned odor alone will activate the common core areas (pink boxes) which trigger motivation and integrate information (yellow circle) about males that bear the CS. Serotonin (5-HT) and endocannabinoid (CB) release and binding in the cortex, limbic structures, and hypothalamus, is associated with an inhibition of the excitatory mechanisms. In animals with a well-developed visual system, like humans, visual CSs likely provide the dominant priming cues, although auditory and olfactory cues should not be overlooked.

CONCLUSION

The study of sexual behavior in animals and humans has come a very long way since the mechanisms of sexual arousal and copulatory responding, and their hormonal and rudimentary neural bases, began to be studied in the 1960s. In particular, the advent of PDE-5 inhibitors for the treatment of erectile dysfunction in men opened up new vistas in the pharmacology of sexual behavior that thrust previous "basic science" studies of the role of different neurotransmitters in sexual behavior into the light of translational clinical science relating to human sexual function and dysfunction. Animal models of a variety of human sexual responses were determined that had predictive validity.[31] It became clear from brain imaging studies in a variety of species using immediate-early gene expression as a measure of activation, and from fMRI or PET studies as measures of activation in humans that a conserved set of neural pathways and neurochemical systems exist in vertebrates to excite and inhibit sexual behavior. Some of those systems are general, and modulate responses to all unconditioned and conditioned excitatory stimuli (e.g., the mesolimbic DA system and its role in incentive motivation) or inhibitory stimuli (e.g., the mPFC and other cortical systems that mediate behavioral inhibition as part of executive function). Other systems are more specific and mediate the autonomic control of genital arousal and the sexual approach and solicitation behaviors that females display as ovulation approaches. Those pathways share functions for parental behavior, feeding, and drug addiction although some, like feeding and sex, may be mutually inhibitory. To what extent do drugs of abuse utilize and usurp the sex and bonding pathways? Understanding the interrelation of these systems, how they are activated by hormones and/or experience, and how experience can override hormonal priming, is a very promising avenue of cross-translational research. Understanding the similarities of bonding to addiction may well prove useful in the treatment of drug abuse or other obsessive-compulsive addictions.

New data always raise new questions but also reframe some very old ones in the literature that have never been resolved. Several of these are outlined below.

What Can Studies of Female Sexual Behavior in Other Species Tell Us About Hormonal Influences on Sexuality in Women?

Much has been learned concerning the neuroendocrine processes and cellular mechanisms by which steroid hormones influence reproductive behaviors in rodents and other animals. Although cellular studies in humans are presently impossible to perform, mechanistic studies in rodents may provide clues about the

neuroendocrine mechanisms by which hormones act and interact in the brain to influence behavior in all species, including humans. A number of basic principles have been derived from work in nonhuman species. For example, these studies demonstrate the importance of considering the timing of hormone treatments, the dosage of hormone, specific hormone used within a particular class of hormones, form of hormone (e.g., long-acting esterified estradiol or nonesterified estradiol), interactions between hormones, the role of steroid receptor coactivators, route of administration, peripheral factors that may influence hormonal response, and the possible mechanisms of action by which hormones and other factors may influence hormone action and subsequently, sexual behaviors.[687]

These major advances in our knowledge belie a continuing gap in our understanding. In particular, scientists are usually forced to measure either behavior, neurochemistry, electrophysiology or neuronal structure, often in a limited time window or with a specific neuroanatomical focus. A major frontier is to understand the causal sequence of these dynamic, network-wide events. Another major frontier is to understand the relative contribution of each of these hormone-induced changes to behavior, given that so many systems are being regulated simultaneously.

At a more granular level of analysis, recent work has identified different cell types in the VMH; however, the wiring diagram for these neurons is unclear. A better understanding of the neurochemistry and connectivity of these neurons would explain in a concrete manner the apparent excitatory and inhibitory controls of lordosis behavior. At present, the functional significance of the diverse effects of estradiol on the dendritic arbors of these cell types is unclear. Detailed knowledge of VMH microcircuitry would help explain how and to what end estradiol exerts these cell-type-specific effects. Furthermore, our present understanding of estradiol-induced changes in VMH dendrite structure is largely divorced from our knowledge of changes in neurochemistry and electrophysiology. A fascinating future direction will be to determine the interplay between changes in synaptic connections and neurotransmission.

What is the Nature of Female Orgasm?

A critical question that continues to generate controversy concerns the existence of a "G-Spot" that when stimulated properly leads to a "deep vaginal orgasm".[688] Still others argue that such an anatomical entity does not exist[689] citing clinical or case studies of women who have never found theirs despite trying. It may well be the case that not all vaginas are constructed the same way, and that internal sensory inputs come in various sizes and shapes. The "G-Spot" may correspond to an internal portion of the clitoris that has differential sensitivity in different women. It is also the case that experience with external CLS as a sole source of sexual stimulation and orgasm may well preclude the exploration of other stimulation points, especially those that might be hard to get to and are never stimulated adequately by a sex partner. fMRI studies of external and internal clitoral/G-Spot stimulation and subjective awareness could help to solve this issue. Indeed, Komisaruk and colleagues have used fMRI to examine brain activation in women self-stimulating to orgasm[690] and it would appear that orgasms can be induced from external clitoral, internal clitoral, and/or cervical stimulation,[691] despite differences in the areas of activation. How these differ in subjective quality could be examined using validated measures such as Orgasm Rating Scale.[692]

A similar problem exists in the animal literature. Although it is clear that VCS and not CLS potentiates estrus termination, it is not known whether paced CLS, VCS, or both, lead to the reward state necessary for the induction of CPP and/or partner preference. Moreover, it has not been established that the penis of the male rat actually makes contact with the cervix during intromission, although the ejaculatory plug of the male forms around the cervix and pubic bone, which would provide intense and continuous VCS when it occurs and until the plug is removed from the vagina. However, the rate and duration of intromissions is higher in paced vs nonpaced conditions.[523] This, and not the presence or absence of ejaculations, is key in the induction of estrus termination and in the neuroendocrine responses (e.g., nightly prolactin surges) that facilitate pregnancy. Erskine[74] has argued that paced copulation results in stronger intromission thrusts, which may well stimulate both the clitoris and cervix.

How Does Awareness of Sexual Incentives Change across the Menstrual Cycle?

More attention needs to be paid to the menstrual cycle and how it affects women's reactions to sexual incentive cues. These could build upon and extend current knowledge about autonomic, emotional, and cognitive changes across the cycle as they relate to the perception of, and reaction to, external sexual stimuli. Those perceptions could then be examined in terms of brain activation following the presentation of stimuli through goggles worn by the subjects. In this vein, cognitive tasks that examine changes in relative attention toward explicitly sexual visual cues presented either above or below conscious awareness could be used in conjunction with physiological measures of sexual arousal to examine how the two are altered across the cycle (see below).

Will There be Drug Treatments for Sexual Arousal, Desire, and Orgasm Disorders?

Disorders of sexual arousal, desire, and orgasm affect a sizable proportion of pre- and post-menopausal women world-wide depending on the criteria used to define the disorder.[693] This can occur as a function of hormone or neurochemical imbalance, genetic "proneness" to inhibition, depression and other mental illness, and the use of oral contraceptives that generate negative steroid feedback.

As noted above, the development of treatments for sexual disorders in women has benefited greatly from preclinical analyses provided by animal studies. Such studies can examine mechanisms at different levels of analysis (e.g., neuropharmacological to molecular) in ways that simply cannot be done in humans. This requires a complete analysis of the behavior of the animal "models", and some degree of predictive validity that what is being observed in the particular animal model corresponds to the sexual process that requires treatment. In turn, this requires a conceptual understanding of the functional endpoint. For example, approach and solicitation in female rats or macaques serves the same purpose as approach, flirtations, and maintenance of eye-gaze in women who have found someone sexually alluring, This is a critical behavioral juncture: in those with desire disorders such behavior simply does not occur. In extreme cases it is not even thought of. What remains in many women is arousal without a proper context and no sexual incentive to blame it on. Others lose the arousal component as well. And of course, without this there is little chance of sexual gratification, leading to sexual interactions that are likely aversive. This may especially be the case if the woman is prone to inhibition (e.g., Ref. 694).

In some women this is remedied easily by psychodynamic therapies, whereas in others it is not. Such women may well have a physiological blunting of response that is due to hormonal or neurochemical systems that either are no longer operating properly to excite arousal and desire (as may occur in many women during and after menopause), or overactive inhibitory systems that have come online for a variety of reasons, some of which may be genetic and due to an increased sensitivity to sadness and depression (e.g., overexpression of the long allele form of the promoter regions of the serotonin transporter gene[695]).

The discovery of potential pharmacological treatments for arousal and desire disorders in women have been largely serendipitous, with the prosexual effects of the MC-4 receptor agonist bremelanotide discovered during clinical trials of the potential tanning drug MT-II[696] and the sexual effects of flibanserin discovered during its trials as a potential antidepressant.[642] The potential of testosterone as a therapy for the treatment of sexual arousal and desire disorders after menopause was first shown in studies where it was added as an adjunct to estradiol.[138] Current treatments with testosterone include a transdermal testosterone patch (Intrinsa) and labial gel (Libigel), both of which produce rather continuous penetration of testosterone into the circulation. Although these have positive efficacy in the treatment of arousal and desire disorders,[697] they do not mimic the normal testosterone rise during ovulation.[698] The efficacy of applying testosterone as a sublingual bolus was advanced by Tuiten et al.[699] and recently shown in clinical trials that applied it in conjunction with a PDE-5 inhibitor or 5-HT1A agonist to treat hypoactive desire induced by either a "bottom-up" lack of genital sensitivity[700] or an abundance of "top down" inhibition over sexual incentive cues.[694] The first effect has been modeled in female rats.[71] The dose of testosterone is extremely small, making it likely that its action is only in the brain. The PDE-5 inhibitor acts in the periphery to relax smooth muscle in the genitals, allowing for more rapid and complete engorgement. Approximately 4h after administration, the brain is "ready" for sex, having undergone genomic changes induced presumably by testosterone that allow both genital arousal and sexual incentive stimuli to be registered and integrated. Essentially this "tricks" the brain into thinking that ovulation has just occurred. The combination is reported to be well tolerated and devoid of untoward side-effects in a number of treatment regimens. A major advantage is that the combination can be taken "on demand" prior to sexual activity.

It is impossible to know at this point whether any of these drugs will be approved for the treatment of hypoactive sexual desire, interest, and/or arousal. However, as work continues to enhance our understanding of the neurochemical systems involved in sexual excitation and inhibition, it is likely that other drugs will be subjected to clinical trials. It has been predicted that combined pharmacological and traditional talk therapies will have better efficacy than either alone.[701] However it is not yet known whether some women, pre- or post-menopausal, will retain their restoration of sexual arousal and desire if the pharmacological treatment is discontinued. All of this could be modeled in rats or other species using appropriate behavioral analyses. It will also become vitally important to understand how testosterone or other androgens are working in the female brain. Although a good starting point is to examine whether they operate on classic intracellular or membrane bound receptors, it may also be the case that they are aromatized into estrogens to induce their actions. This will be important in determining how testosterone sets up the neurochemical substrates that bring sexual incentive cues into conscious awareness in the female brain. Likewise, a greater understanding of the roles played by OT and prolactin in women is critical in helping to elucidate

the mechanisms of orgasm and its aftermath. This may help to differentiate women with orgasm difficulties who have either never experienced them or experienced them fleetingly vs those that have sluggish brain and/or autonomic reactions to sexual stimulation.

How Does Sexual Responding Change with Age?

As the so-called "Baby Boomer" generation is aging and millions of women world-wide are reaching menopause and beyond, more research must be focused on how the aging female brain and body change in response to the hormonally unstable and ultimately hypogonadal condition that menopause engages (see Chapter 37). Women experience changes in cognition and emotional reactions, in addition to bone density and fat deposition as a function of altered metabolism. Sexuality changes during aging as a function of experience and expectation, relationship status, hormonal alterations, and changes in genital sensitivity. Very little is understood about age-related changes in the brains of women in regards to sexuality, and little has been studied in this regard in animals. Aging brings about cardiovascular and metabolic changes associated with conditions such as diabetes and hypertension, which can blunt sexual arousal and desire in both men and women.[35] The treatments for those conditions also have sexual "side effects" that blunt sexual arousal and possibly desire and orgasm,[35] so treatments that restore sexual function must be able to operate independently of those mechanisms and not exacerbate them.

Can We Infer Mechanisms in Females from Similar Mechanisms in Males?

There is no question that substantial sex differences exist in morphology and brain neurochemical function[702] that underlie differences between females and males in response to sexual stimuli,[703] motivation,[704] control of pituitary hormone systems in rodents,[705] pain,[706] and mental health.[707] Some of these mechanisms have their origin in the initial sexual differentiation that occurs in the neonatal brain,[708,709] whereas others do not.[710,711] In particular, McCarthy et al.[711] note that a common strategy in the experimental approach taken by many neuroendocrinologists is "after I understand the phenomenon in males, I'll check whether it's there in females". This approach has also been taken in understanding the hemodynamic of sexual arousal in men and women, often with the assumption that anything that induces penile erection in men should also work the same way for the induction of labial, clitoral, and vaginal erection in women. Certainly PDE-5 inhibitors do increase vaginal vasocongestion but many women are unaware of that, leading to the phenomenon of "discordance" between subjective reports of sexual arousal and physiological measures of such arousal.[151] Such discordance between subjective and physiological sexual arousal is not observed in men unless they have consumed a threshold dose of alcohol that blunts erection but disinhibits subjective arousal and desire.[13,712] There may be several reasons for this. Sex differences likely exist in the cellular response to cyclic vs continuous gonadal hormone output; in the reaction to context; and in individual responses to preexisting stressors. Individual differences also exist in terms of experiences with, and attitudes toward, different types of erotic stimuli that are presented to provoke a genital response. These sex and individual differences conspire to create differences in conscious sexual response which is reflected in brain activation (e.g., laterality in amygdala responsivity[703]). Although it is difficult to find sex differences in the human sexual response cycle, other than the ability of some women to have multiple orgasms relative to men's inability to do so, there are obvious sex differences in the behavior of female and male rodents. Those differences are typically "reverse-engineered" back to differences in differentiation of brain and body, and to specific differences in brain area, for example, the sexually dimorphic nucleus of the preoptic area. Indeed, lesions of the POA in male rats disrupt erection, ejaculation, and mounting behavior, whereas lesions in female rats disrupt solicitations and pacing, and may affect the hemodynamics of clitoral engorgement or the ability of genital stimulation (clitoral and/or cervical) to induce reward. At one level, those functions and the behaviors they subserve seem very different. At another, however, they both involve the responses to genital stimulation that bring about direct sexual contact: solicitations and pacing in females and mounting in males. In fact, in the human brain activation literature, there is an overwhelming preopoderance of sex *similarities* in regional responses to erotic visual stimuli.[45,713] Thus, in rats, the same regions may respond similarly to sexual stimulation but induce output that appears different at a behavioral level. In humans, the behavioral differences may well have disappeared, or been impinged upon by a greater executive cortical/cognitive control over sexual behavior that is mediated more by context and social learning. However, rats clearly have the capacity to make both Pavlovian (stimulus–stimulus) and operant (response-reinforcer) associations between sexual stimuli and sexual responses that can span first- and second-order conditioning, even to the point of conditioning of sexual fetishes for rodent tethering jackets.[28,714]

Context is also an important component of sexual behavior in both female and male rats.[50,70,668] It may well be the case that sex similarities exist in rats, as they do in humans, especially at "higher" emotional and cognitive processing levels that include cortical processing. It

is the case that human neuropsychology and brain imaging studies on incentive sexual responses have focused on cortical and limbic structures, usually with a mention that the hypothalamus is or is not activated, whereas animal studies have focused largely on hypothalamic and some limbic activation of immediate-early gene products, or microdialysis/voltammetric analyses of neurotransmitter turnover. Rarely do neuroendocrinologists study the cerebral cortex, nor do neuropsychologists delve into the hypothalamus, and this confounds our understanding. In fact, analysis of both animal and human brain activation to sexual stimulation reveals nearly identical cortical responses,[64] suggesting that even "higher processes" have been conserved, and that the sexual brain is not simply reducible to hypothalamic processing.

How Does Environmental Context Modulate Hormonal Action on Female Sexual Behavior?

A number of examples were given earlier of nonsteroid hormone factors that can influence steroid receptors, and subsequently behavioral response. In addition, we discussed the idea that stimuli from the environment (mating stimulation) can activate steroid receptors indirectly by ligand-independent activation. This mechanism provides a means by which an array of environmental influences could alter sexual response. Do these mechanisms come into play in the real world of a wild rat? Do attempted mounts by conspecifics or other environmental factors alter the timing of the commencement of sexual behaviors? And might particular contexts or types of stimulation activate steroid receptors in women thus influencing sexual response? It is also important to consider whether incentive cues associated with sexual reward alter steroid ligand–receptor interactions and whether such changes, if any, can compensate for the loss of hormonal priming that occurs in hypogonadal individuals.

Final Remarks

Our understanding of the neurobiology of female sexual function is fast reaching a level of depth and sophistication that rivals that of male sexual function. Animal models of human female sexual function and dysfunction have been proposed that emphasize reward-related learning and cognitive assessment of context. Within these new paradigms, the role of ovarian hormones, gene expression, neurochemical mediation, and the impact of brain lesions (both surgical and accidental) are beginning to be assessed. The sexual brain integrates sensory and hormonal inputs to the hypothalamus to activate incentive motivation and emotional responses in limbic structures. These are under cortical control to inhibit unnecessary or competing responses, or indeed to inhibit sexual responding altogether in inappropriate contexts or situations. The combined actions of these systems optimize female sexual responding, so that what is most rewarding is also likely to be the most reproductively efficient. That these systems are essentially conserved in all mammals—and perhaps all vertebrates—is a testament to our continued survival on this planet, survival that depends critically on the ability of females to approach, solicit, pace, and engage in rewarding sexual activities under their control.

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CHAPTER

51

Parenting Behavior

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INTRODUCTION

The reproductive venture in mammals typically begins with the task of choosing a mate and is followed by the copulatory interactions that culminate in the joining of ova and sperm. For almost all mammals, reproduction and its behavioral correlates do not end there. Most mothers give birth to young that are unprepared for an independent life ex utero, thus requiring that the neonates receive prolonged and intense parental care for their sheer survival. Caregiving by most animals is highly sexually dimorphic,[1,2] so for the majority of offspring this nurturance is provided by their mothers and is termed *maternal behavior*. In animals that have a relatively rare reproductive strategy involving biparental care, fathers will provide *paternal behavior*. In addition, caregiving may not be restricted to the parents and can be displayed by other willing participants, including the neonate's older siblings or other close relatives, who provide beneficial *alloparental behavior*.

Potential caregivers are often not spontaneously interested in young or may not be able to adequately care for them even if interested in doing so. Through the process of pregnancy and parturition, a profound neurobehavioral transition occurs in maternally inexperienced female mammals such that any clumsiness or indifference to neonates, or even outright aggression toward them, is replaced by skillful and tender caregiving. Of course, caregivers other than biological mothers do not gestate and give birth to offspring, so there must be alternative means that promote their nurturant behaviors.

The literature detailing the physiology of mammalian parental behaviors has been reviewed numerous times with tremendous thought and detail. The goal of this chapter is not to again exhaustively review this large literature, and we refer readers elsewhere for details not found herein.[3,4] We instead highlight what we view as the major scientific advances in our understanding of the endocrinology, sensory regulation, and neurobiology of nurturant behaviors displayed by mothers, fathers, and sometimes alloparental "helpers." Similar to the reductionistic study of most mammalian behaviors, the physiology of parenting is best understood in laboratory rodents. This is justified by their exceptionally successful breeding within a laboratory environment, the broad social acceptance for their use in basic research, a wealth of existing knowledge about their endocrinology and neurobiology, and the important belief that such an evolutionarily conserved behavior as parenting will have similar biological underpinning across mammals. Indeed, sufficient investigation of nonrodent parents, including sheep, rabbits, and primates, provides richness to this literature and ample data that allow one to detect potential universals, as well as species-specific mechanisms, involved in this biologically complex, incredibly motivated, and evolutionarily essential mammalian social behavior.

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Historical Perspective

Studying the biological mechanisms underlying parental behavior has a century-long history. Some of the earliest research on this topic refers to observational or experimental work on the endocrine, sensory, or neural basis of copulation in rodents and other small mammals; thus, we can presume that the study of parenting behavior arose from and was informed by the study of mating behavior. Cross-fertilization between these fields still occurs today, which is understandable given the discrete set of sensory, endocrine, and neurobiological faculties that animals have available to conduct a wide range of reproductive and other social activities. Contemplating the similarities or differences among behaviors, such as parenting and mating, will continue to provide valuable insight into the biological mechanisms underlying them both.[5,6]

Questions asked in the very early studies of mothering were quite similar to those asked today. For example, the search for bloodborne factors that are released during pregnancy or lactation and involved in maternal caregiving was studied as early as 1925 by Stone,[7] who surgically conjoined virgin and pregnant female rats in hopes of finding accelerated maternal responding in the conjoined virgin. Stone's study was unsuccessful, but reasonable given that parabiotic methods had previously shown that bloodborne factors could travel from one animal to affect the physiology of another.[8] Such parabiotic studies were resurrected by Terkel and Rosenblatt almost 50 years later, who did find enhanced maternal responding in the conjoined virgins (see the section Hormones Most Significant for the Onset of Maternal Behavior in Rodents, Sheep, and Humans).[9,10] The 1920s also saw reports that particular patterns of ovarian activity were correlated with mating in female rats and that ovarian hormones could induce copulation when exogenously administered.[11,12] Either in response to these studies, or simply consistent with the zeitgeist, a flurry of studies emerged soon thereafter reporting that mothering would be displayed by dogs given extracts from the urine of pregnant women[13] and that maternal behavior could be seen in a nulliparous monkey[14] or in virgin rats[15] given crude extracts of the pituitary gland.

The next few decades saw numerous, but often methodologically compromised and contradictory, studies attempting to pinpoint the ovarian and pituitary secretions that could induce or inhibit mothering. Some reported that ovarian hormones could only inhibit mothering,[16] while others found that seemingly any ovarian, pituitary, or thyroid hormone could promote maternal behavior when given alone or in combination.[17] Buried amongst such discrepant results were findings that later became fundamental concepts in the current field studying parenting behavior. For example, prolactin (PRL) has been known for decades to promote the onset of maternal behavior in ovarian hormone–primed nulliparous rats.[18] Also, it has long been known that after hormones promote the onset of maternal behavior in many species, endocrine factors are unnecessary for maintaining the behavior (through a process termed "eroticization" by Steinach[19]) such that ovariectomy or hypophysectomy has little effect on postpartum mothers' general interest in pups.[15,20] Early studies also demonstrated the still often-used paradigm of maternal "sensitization" (see the section Hormones Most Significant for the Onset of Maternal Behavior), in which nulliparous rats with no exogenous hormone treatment and no interest in pups begin to perform caregiving behaviors after repeated exposure to neonates.[15,21] Some early misconceptions about the endocrinology of mothering were not clarified, however, until as late as the 1960s. These included that neither PRL nor progesterone could alone promote maternal behavior in virgin female rats and instead required the assistance of other endocrine factors.[22–24] One of these factors is now known to be estradiol, although at least two studies from the 1960s (which can retrospectively be seen as the beginning of the "modern era" for the biological study of maternal behavior) could not demonstrate estradiol's effects, probably because the temporal sequences of the exogenous hormones given were endocrinologically incorrect (see the section Hormones Most Significant for the Onset of Maternal Behavior).[24,25]

Extant research on the hormonal regulation of caregiving behaviors shown by male mammals also began almost a century ago, but often had the goal of inducing maternal-like behaviors in species of males that were not naturally paternal. These studies included observations of female-like interest in pups by guinea pigs and rats that were peripubertally castrated and given ovarian grafts[19,26] and the display of maternal-like behavior in male rats given anterior pituitary implants during adulthood.[27] Brown has more recently criticized this strategy as misguided for understanding paternal behavior in any naturally biparental species because a female-oriented focus leads one to incorrectly assume that the endocrine and neural factors underlying maternal behavior are similarly responsible for paternal behaviors in fathers.[28] As far as we can determine, the earliest work manipulating the endocrine system of any spontaneously paternal male mammal was studies from the 1930s that found no effects of hypophysectomy on the paternal behavior of male laboratory mice.[20] Studies of the steroid hormones involved in a paternal male primate came much later, with Wilson and Vessey's 1968[29] report that castrated male rhesus macaques (*Macaca mulatta*)—albeit a species that is still not particularly paternal in natural environments—are particularly prone to exhibit paternal behaviors and Alexander's 1970[30] suggestion that the androgen withdrawal occurring across seasons in the

more naturally paternal male Japanese macaque (*Macaca fuscata*) was associated with males' increased paternal play with young. As seen in the section Hormones Most Significant for Paternal Behaviors, endocrinological studies of the now exemplar rodent models used to study the natural occurrence of fathering—California mice (*Peromyscus californicus*), prairie voles (*Microtus ochrogaster*), and dwarf hamsters (*Phodopus campbelli*)—began to emerge just less than two decades ago.

With regard to the sensory control of parenting, there was evidence 80 years ago that blinding or anosmia had little effect on nest building or other maternal behaviors in postpartum rats. Beach and Jaynes[32] later systematically provided many additional insights into this question, again partly drawing on previous work studying the sensory control of copulatory behavior in rats and other small mammals.[33,34] By studying the effects of individual and multiple desensitizations on retrieval of pups, Beach and Jaynes laid the groundwork for our understanding that maternal responding in rats was under "multisensory control." More recent studies re-examining the sensory control of retrieval and other maternal behaviors in primiparous laboratory rats, and the responses of human mothers to infant sensory cues, support many of Beach and Jayne's early conclusions that no single sensory modality is indispensable for most mothering behaviors (see the section Sensory Control of Maternal Care).

Starting in the 1930s, electrolytic or aspiration lesions were employed to study the central nervous system control of maternal behavior, but these studies produced discrepant results for decades.[35] The cerebral cortex was the first focus of this work and, once again, it took some lead from research that had unsuccessfully sought to find the cortical "sexual center" in male rabbits.[36] In his studies examining cortical control of mothering as an exemplar innate behavior, Beach found that the amount of cortical tissue loss was proportional to the temporospatial disorganization of the females' maternal behaviors but that the location of the cortical loss was mostly irrelevant.[37,38] Around the same time, Stone[39] and Davis[40] also reported that substantial decortication abolished maternal behavior. Later experiments did indicate cortical specificity for mothering,[41] though, and suggested that earlier cortical lesion effects on maternal behavior were partly due to subcortical damage.[42,43]

The first clear evidence for a subcortical influence on caregiving behaviors came from Fisher in 1956,[44] who described how nest building and sometimes retrieval and licking the pups could be elicited in a small number of male rats that received infusions of sodium testosterone sulfate into the medial, but not lateral, preoptic area. Prepartum lesions of the ventromedial preoptic area were later reported to have no consequence on postpartum maternal behavior, while ventromedial nucleus lesions elicited infanticide.[45] Given what we now know about the roles of the preoptic area and ventromedial hypothalamus in maternal behavior (see the section Brain Control of Maternal Behaviors), both of these findings would be surprising if reported today. Other studies around this time found that lesions of the lateral hypothalamus, septum, hippocampus, or mammillary bodies disrupted mothering in female rats[42,43,46,47] and that lesions of the septum and cingulate cortex could sometimes produce negative consequences on mothering in postpartum mice.[48,49] It will be seen in this chapter that significant advances in experimental methodology—including more widespread use of and greater sophistication in neuroanatomical tracing, the ability to use genomic responses or regional oxygen utilization to visualize the activity of multiple brain sites simultaneously, and the development of techniques to manipulate the rodent genome—have permitted the field to move away from using lesions to conduct this type of site-by-site analysis of the neural structures required for parental behavior and instead provide a more network- or integrative systems-level understanding of how the brain controls caregiving behaviors (see the section Brain Control of Maternal Behaviors).

COMPONENTS OF PARENTAL CARE

Successful parenting results from parents' increased attraction to infants, the perfection of infant-directed caregiving behaviors, and modification of existing capabilities that are not even oriented toward the offspring but nonetheless promote their survival. Some of these modifications in non-offspring directed behaviors include blunted emotional reactivity that may help parents focus on the needs of their young,[50] enhanced cognitive skills that may optimize parental responding to the neonates and other aspects of the environment,[51] and aggressiveness toward potentially infanticidal intruders. This last modification is outside the scope of this review, so we refer readers elsewhere for information about the hormonal and neural control of maternal aggression.[52,53]

It is important to begin the discussion about the components of parental care by first highlighting that the nature of parental attraction to infants differs considerably among species. Polytocous rodents such as laboratory rats, mice, and rabbits that give birth to many offspring can discriminate between their own and unrelated pups, but form no exclusive bonds with them. In laboratory environments, these mothers will indiscriminately care for any conspecific or even nonconspecific neonates. Such generous mothering could be a laboratory artifact, but

instead might be relevant in natural environments when lactating mothers communally nest.[54,55] In contrast to these rodents, maternal sheep form an impressively selective bond with their offspring at parturition that prevents foster caregiving. Unlike either example, most human mothers form an intense bond with their infants, but this bond is not exclusive and the possibility of caregiving toward other infants of course remains.

There are also considerable differences among species in their parental repertoires, which often lies in the fact that parent–offspring interactions are necessarily dyadic, involving reciprocal stimulation between the participants,[56] so adult responses tremendously depend on the characteristics of the offspring. These include the newborn's neurobehavioral competency for independent survival. Neurobehaviorally undeveloped neonates (termed *altricial offspring*) are often offspring of small, short-gestationed mammals, such as rodents, rabbits, dogs, and cats; at birth, they have a limited ability to thermoregulate, locomote, see, or hear. Even more extreme are the altricial young of some species that appear almost embryonic at birth, such as some marsupials that are born after an unusually brief gestation and spend their early postnatal life within their mother's pouch to seemingly finish up what was unaccomplished during in utero development.[57] In contrast to these altricial offspring, relatively neurobehaviorally developed neonates (termed *precocial offspring*) often arise from longer gestating mammals such as sheep, horses, and guinea pigs; they are born furred, with eyes wide open, and are surprisingly mobile. Such differences between altricial and precocial young are relevant for understanding the relationships among the hormonal profiles of nonhuman animals, that of our own species, and females' caregiving behaviors. One salient example is that while both the sheep (~147 days) and human (~280 days) have lengthy pregnancies, sheep give birth to precocious offspring, whereas human offspring are relatively altricial. The durations of hormone exposure in these species are more similar to each other than, say, to that of laboratory rats, but maternal interactions with young at parturition differ tremendously. Thus, the hormonal patterns during and after pregnancy, and the developmental state of the young at birth, must all be considered to accurately uncover the neurobiological mechanisms driving maternal care in any given species.

Based on their developmental stage, one might expect that maternal care is frequent and protracted toward altricial young, but infrequent and brief toward precocious young. This is not necessarily the case. Startlingly, whereas altricial rat pups are in the nest with their mothers for 70–90% of the time,[58] altricial rabbit pups receive only 3 min of maternal care each day.[59] Similarly, precocious ungulates are either "cached" away and visited by the mother just a few times per day[60] or are members of species that follow close behind their mothers and remain within steps of her most of the time.[61] Species differences in the fat, protein, and water content of maternal milk allow such variation in the frequency of mammalian caregiving to exist.[62]

Regardless of the neonate's developmental stage at birth, offspring-directed behaviors may seem to be a seamless thread of activities, but a closer look reveals that they are instead a collection of behavioral routines and subroutines that are appropriate to study both individually and collectively. This study is often facilitated by careful observation of the individual behaviors displayed, and then grouping them into meaningful categories of activities that might involve particular parts of the parents' bodies, are most often temporally associated, or appear to have similar or interdependent purposes. This task can be difficult because not all mammalian parents display the same repertoire of behaviors and universal categorization of parental activities may be impossible. Even so, many researchers have distinguished *active* parental behaviors from *inactive* parental behaviors. Active parental behaviors include many of the hallmarks of caregiving, including establishing a place where mother–offspring interactions can occur, which is often called nest building. For many animals, including most small rodents with altricial young, this involves transporting suitable materials to a central location and manipulating them into a safe and secure nest. Active parenting also often involves carrying displaced offspring from one place to another using the mouth, hands, or arms, or more passively allowing the offspring to cling to the parent's fur to hitch a ride. Carrying or retrieving neonates is easily quantifiable in both the field and laboratory, but it is important to note that its propensity is not universal. It is mostly unnecessary and even impossible in precocious species with heavy or highly mobile young, and it is also rare even in some relatively altricial species. These include prairie voles (*M. ochrogaster*), whose pups are born with teeth that help maintain attachment to the mother,[63] and rabbits who nurse at the bottom of a deep burrow from which the pups cannot easily escape.[64] Cleaning the offspring is another common active parental behavior and is performed by nonprimates by using the mouth to lick or gently nibble, but in primates it also involves the parents' hands.

In many mammals, these active maternal behaviors are often performed in preparation for prolonged periods of inactive nursing, the behaviors involved in transferring mother's milk to the infants. Lactation (i.e., milk production and letdown) is not a behavior, and there is a tremendous amount of information known about this physiological process that can be found elsewhere. (see Chapter 46; and Refs 65,66). With regard to nursing behavior, in response to offspring suckling some mothers undergo a transition from a highly active state to one of inactivity and quiescence, which in laboratory rats, cats, and primates

involves slow-wave sleep.[67–69] Only during these periods of quiescence does milk letdown occur in rats.[70] In rabbits and pigs, suckling elicits behavioral inactivity, but it is associated with increased cortical firing rather than a depression.[71,72] Many four-legged mothers that are simultaneously nursing multiple offspring will assume a distinctive posture, termed *kyphosis*,[73] which involves limb rigidity and an upward flexion of the spinal column. This nursing posture provides additional room for young to breathe, move, and suckle while underneath their mother. Kyphosis is more likely to occur earlier in lactation than later and is the optimal position for milk letdown during early lactation in laboratory rats.[74] In addition to kyphosis, mothers can be readily observed lying prone on top of the young with little limb support or found lying in a supine position next to or under the pups. These latter postures may be especially common when mothers are either fatigued by the rigidity of kyphosis, when offspring are too large for the entire litter to fit under her, or when older pups initiate nursing from a recumbent mother.[73]

CONSIDERATIONS FOR UNDERSTANDING THE HORMONAL REGULATION OF MATERNAL CARE

The hormonal profile of pregnancy and the peripartum period is crucial for supporting the developing fetus, while at the same time prepares "the expectant brain" and the periphery for the expression of maternal behavior and other required physiological processes such as lactation (see Chapter 44). Extensive experimental evidence has demonstrated that estradiol, progesterone, PRL, and sometimes oxytocin (OT) can play crucial roles in the onset of maternal behavior in rodents and ungulates, with the addition of cortisol important in primates. A remarkable feature of the hormonal profiles over a wide range of species is the similarity of the hormones and their temporal course during pregnancy, parturition, and the postpartum period. Another remarkable feature is that not only do the maternal ovaries, adrenals, and anterior pituitary act as hormonal sources during pregnancy, as they do during all reproductive phases, but the placenta and fetus individually and together are also powerful sources of hormones unique to pregnancy that can act peripherally and perhaps centrally.

Basic information about this hormonal profile is essential for understanding the state changes in the female brain that increase maternal motivation and her readiness to provide care to the young. Because these hormones act on the periphery and brain via their respective families of hormone receptors, it is also critically important to understand how reproductive state alters hormone receptor expression, affects the dominance of particular subcategories of receptors, and how these receptors are influenced by their ligands to influence neuronal activity and maternal responding.

In pregnant and postpartum mammals, the hormonal profiles of rodents and humans have been the best studied, but some additional models including sheep have significantly added to our understanding of this topic. Each nonhuman animal model possesses different features of face, construct, and predictive validity for the hormonal basis of parenting in humans, but no single model provides a complete picture homologous to what is known from humans. Even so, each model holds uniquely useful characteristics for examining the emergence and continuation of maternal behavior. For example, a valuable feature of the laboratory rat model is that while rats do recognize their own young, their assiduous and rapid care for the young of other individuals facilitates many laboratory studies. On the other hand, a crucial and fascinating feature of caregiving in sheep is the selective mother–offspring bond, which is based on individual recognition and memory of lamb cues, and which may inform some aspects of maternal bonding in humans.

Correlating hormone levels in the pregnant and parturient female with the expression of maternal behaviors is an important approach to examining the hormonal basis for maternal behavior in all species examined, and really the only approach available to do so in humans. When considering the major changes in the hormonal milieu for the three exemplar species discussed in detail below, it is absolutely crucial to consider the extent to which the maternal brain is bathed in this milieu: that is, whether the blood–brain barrier does or does not prevent the hormones of interest from reaching the brain. It is generally accepted that steroid hormones readily access the brain, but most peptide hormones of peripheral origin are more highly regulated or almost completely restricted from access. Some peptides, including those in the PRL family, gain access via peptide-specific transporter systems that result in cerebrospinal fluid levels being highly correlated with plasma levels.[75] However, less than 3% of most other peripherally circulating peptides without such transporter systems, including OT and arginine–vasopressin (AVP), enter the brain.[76] While there is limited evidence that the blood–brain barrier is not altered by pregnancy,[77] the access of such peptides to the brain during this time has not been experimentally examined in detail, so great caution is warranted when attempting to extrapolate peripheral (plasma and saliva) peptide hormone titers to what may be found in the brain. In addition, the marked advantages of animal models for examining the hormones necessary for the onset of maternal behavior allow experiments that provide causal evidence because the removal of glands

and supplementation of hormones are readily possible. There are substantial limits to such reductionist approaches, however, because disrupting the endocrine and physiological demands of pregnancy, parturition, and lactation can inadvertently confound analysis of the behavioral outcomes of most interest.

The brain itself is also a source of steroid hormones and peptides that act as neuromodulators, including OT and AVP. As would be expected for neuromodulators, site-specific release of OT and AVP has been confirmed and can be independent from plasma levels.[78,79] Although independent brain and peripheral release has been demonstrated, it is also the case that OT and AVP are released intracerebrally at parturition and during suckling in apparent coordination with peripheral release, suggesting a hormonally coordinated linking of central and peripheral events in mothers.[80]

A great deal of recent research on laboratory rats and mice has been devoted to studying how naturally occurring genetic variation among individuals or genetic manipulations reveal important features about the endocrine and other neurochemical bases of mothering. The most gene-specific manipulations for probing the endocrine and neuroendocrine basis of maternal behavior have been carried out with transgenic laboratory mice, and most of these have used global and lifelong knockout (KO) of specific endocrine system genes.[81] It is invaluable to realize that transgenic manipulations of receptors of ligands that mediate the hormonal underpinnings of maternal behavior can have the unintended consequence of altering fertility or the capacity to maintain pregnancy and undergo parturition. This makes it impossible to examine the hormonal and other factors involved in naturally occurring postpartum behavior, but does permit investigation of how these females respond to pups after repeated exposure to them in a maternal sensitization paradigm (described in detail below), which is thought to be non–hormonally controlled. It is also important to recognize that the neurobiological control of maternal-like responses in females that have not undergone pregnancy and parturition could differ from those that have experienced these reproductive events. Furthermore, because different strains of nulliparous rats and mice vary in their baseline maternal responsiveness,[2,82,83] careful consideration of the variability in these baselines is crucial because high spontaneous maternal behavior may be confused with the impact of endocrine system gene KO, which may be subtle in strains with spontaneous maternal caregiving. Thus, there is a heightened requirement in studies of transgenic models to make a faithful comparison of the impact of the genetic manipulation on the baseline postpartum or virgin maternal performance in the same strain. This issue of "background matters" has also been demonstrated for male sexual behavior in gene-disrupted mice,[84] making this a generalizable consideration important for all studies examining the genetics of behavior.

Regardless of the form of genetic or other manipulation, experiments impacting natural postpartum maternal behavior offer the most interpretable data when quantitative information is included about parturition behavior to verify that offspring are delivered alive and cleaned of amniotic membranes, that offspring viability is measured soon after parturition (including litter size and weight in rodents), and that the mothers are lactating normally (including repeated measurement of offspring weight or postmortem mammary gland weights). Because of the dyadic nature of mothering, further crucial inclusions include regularly assessing offspring vigor and replacing any sickly young with healthy foster young of a similar age, and maintaining a species-typical number of offspring for each mother to interact with.

For studies of behavior after global or lifelong knockouts of any protein, it is also necessary to consider the possibility that behavioral resilience or alteration might be due to redundant proteins that take a larger than usual role or compensation by functionally associated molecules that otherwise would not be involved. A further complication is the case in which a ligand normally regulates the expression of its own receptor, for example regulation of the OT receptor by its ligand, but where the receptor is normal even in the absence of OT, suggesting that other molecules have taken over its usual role.[85] Also importantly, the absence of some proteins during development may generate very different functional alterations than if the proteins are absent only during adulthood. While conditional gene inactivation approaches may limit some of these problems by allowing temporal or spatial restriction of the KO or knockin, they bring other issues that have recently been discussed in the context of the androgen receptor KO models.[86,87] Frequently encountered limits of the conditional activation systems include the completeness of the excision (i.e., a knockout that is really a knockdown), the confounding influence of the floxing itself, and the leakiness of Cre activation into off-target cells or tissues. In the best circumstances, proof of effectiveness and target specificity can mitigate these problems, while in the worst cases the KO model being studied does not allow one to make interpretable conclusions.

HORMONES MOST SIGNIFICANT FOR THE ONSET OF MATERNAL BEHAVIOR IN RODENTS, SHEEP, AND HUMANS

Harking back to Stone's early parabiotic studies examining if bloodborne factors were involved in mothering behaviors,[7] Rosenblatt and Terkel reported in 1968 and 1972 that injecting plasma from early-postpartum laboratory rats into virgins, or commingling their

circulatory systems, dramatically reduced the latency to show maternal responding (Figure 51.1).[9,10] Transferring periparturitional blood was by far the most effective. An almost innumerable number of endocrine events occur across the weeks or months of pregnancy and parturition, and up to years of lactation depending on the species. Are they all required for mothering behaviors? Responding to foster pups by laboratory rats increases dramatically in the final days of pregnancy, presumably in preparation for the litter's appearance at parturition.[88] In laboratory rats, the duration of pregnancy before surgically terminating it via Caesarean procedures is positively associated with females' maternal responsiveness, and at least 16 days of rising estrogens and exposure to placental hormones followed by the withdrawal of progesterone is needed for a robust onset of maternal behavior.[89,90] Of course, successful pregnancies normally terminate with parturition, when offspring would naturally first be available to the female. Because some aspects of maternal behavior appear prepartum and are displayed by late-pregnancy terminated rats, the physical processes of parturition are clearly not required for the onset of maternal behavior.

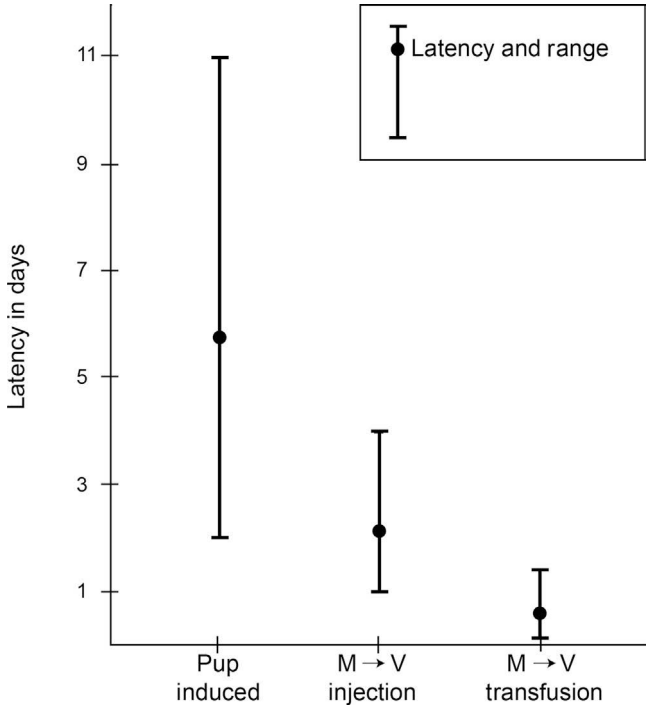


FIGURE 51.1 Mean and range of the number of days of pup exposure for nulliparous female rats to begin retrieving pups. Females were exposed to pups alone (Pup Induced), were exposed to pups after receiving an injection of blood plasma from a recently parturient and maternal female (M→V Injection), or were exposed to pups during and after a 6 h transfusion of blood from a recently parturient and maternal female (M→V Transfusion). Transfusion from a parturient female very rapidly induced retrieval in the nulliparous rats. *Source: Modified from Terkel and Rosenblatt, 1972.*[10]

Even though the onset of mothering does not require the sensory input of parturition, including vaginocervical stimulation, or the ingestion of placentae and amniotic fluid, these factors can still have positive effects on mothering in some laboratory rats.[91,92] Vaginocervical stimulation naturally received during parturition is required, however, for the onset of mothering in many female prairie voles (*M. ochrogaster*)[93] and wild house mice[94] that remain unresponsive to pups or even infanticidal through the end of pregnancy.

In contrast to most laboratory rats, sheep do not become more maternal as parturition approaches and the visceral events of either natural delivery or experimentally produced vaginocervical stimulation are needed for motherhood and development of the mother–young bond. Indeed, epidural anesthesia is a major impediment to the onset of their maternal behavior,[95] and receipt of vaginocervical stimulation must occur within a short time frame before the appearance of a lamb to avoid its total rejection by the ewe.[96] While treatment with exogenous estradiol, progesterone, and central OT can induce maternal responsiveness even without vaginocervical stimulation in ewes, this is much more difficult to achieve than in the rat. In the natural situation, both peripheral and central systems are coordinated by the processes of parturition, and vice versa, for maternal behavior in ewes (discussed further below). In humans, the now common use of Caesarean delivery has revealed that vaginal delivery is not required for their maternal responsiveness, but vaginal delivery is associated with greater maternal neural and behavioral sensitivity to infant cues as well as more positive maternal mental health,[97,98] all perhaps related to the endocrine and other chemical factors released during parturition.

While the hormonally induced changes to the maternal brain instituted by pregnancy and the peripartum period are vital for neural sensitivity to offspring cues and the display of maternal behavior in the early postpartum period, after parturition the presence of hormones becomes progressively less important. The maintenance of maternal behavior is instead regulated by interaction with the offspring, with the dyad becoming an interactive duo that produces changes in maternal behaviors according to the needs of the developing offspring. Indeed, mothers and offspring of probably all mammalian species spend progressively less time with each other as the offspring become more physically and motorically independent.[99,100] A nonhormonal maintenance of maternal behavior is supported by many studies showing that removal of the pituitary, ovary, or adrenal glands does not terminate postpartum maternal behavior.[3] There may be some changes in postpartum maternal behavior after removal of these glands, but they are quite subtle compared to their role in the onset of maternal behavior. For example, adrenalectomy has

little effect on retrieval, nest building, and nursing in postpartum rats, but it does increase how long mothers lick their pups and alters where licking is focused on the pups' bodies.[101] Similarly in the ewe, after postparturitional acceptance of the lamb, hormones are not required for maintaining maternal care and the mother's interaction with the lamb is instead crucial.[102] In humans, postpartum interaction between the mother and infant optimizes ongoing mothering, although there seems to be continuing modulatory roles for maternal OT and cortisol in regulating this interaction.[103,104]

Mothering without Pregnancy or Parturition?

In some species, mothering-like behavior is possible without undergoing pregnancy and parturition—which is sometimes common in parental males, juvenile siblings, or genetically related nulliparous adult females—indicating that there are multiple means by which the neural substrates for parenting can be activated. Most virgin adult and juvenile laboratory rats of both sexes can be experimentally stimulated to perform some maternal behaviors simply by continuously exposing them to pups. Because these caregivers cannot lactate, the health of the foster pups is maintained by once- or twice-daily rotation to lactating surrogates. The emergence of such pup-induced or sensitized maternal behavior in nulliparous female rats usually takes up to 6–10 days of exposure and alters females' behavior from avoiding the pups to avid caregiving. This sensitized maternal caregiving is thought to be hormone independent because it is not prevented or even greatly delayed by removing the adult subject's gonads, pituitary gland, or adrenals before initiating the exposure to pups.[105] The maternal-like behavior of sensitized rats may be impressive, but it does differ from the behavior of naturally parturient females in its deficient retrieval, pup licking, and nursing (Figure 51.2).[106,107] The relationship between sensitized and postpartum maternal behavior has also been compared within subjects, and there is a positive correlation between how rapidly adult nulliparous rats sensitize and how often they later lick their own offspring after giving birth,[108] but there is no relationship between sensitization during juvenile life and postpartum behavior in the same female rats.[109]

Sensitization has also been used in various strains of mice, but both laboratory house mice (*Mus*) and deer mice (*Peromyscus*) have high levels of baseline caregiving, again an important but sometimes overlooked consideration regarding their use for studying the hormonal or neural basis of maternal behavior. There is no evidence that nulliparous sheep are susceptible to any sensitization of maternal behavior, and sensitization has been demonstrated to not be readily achievable in other species, including rabbits.[110]

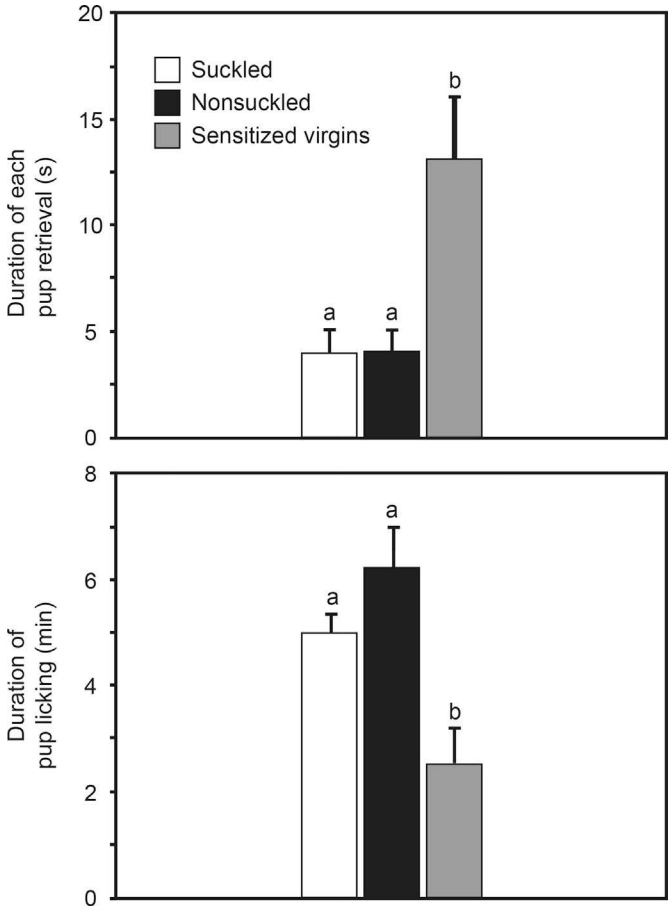


FIGURE 51.2 Duration of time (Mean + SEM) that suckled and nonsuckled postpartum rats, and maternally sensitized nulliparous rats, took to retrieve each pup to the nest (top panel) and spent licking the pups (bottom panel). Different letters above bars indicate significant differences between groups. Maternally sensitized nulliparous rats were deficient in both measures compared to postpartum mothers. *Source: Modified from Lonstein et al., 1999.*[107]

The sensitization paradigm has been combined with knowledge about the hormonal progression of pregnancy to establish that after days 17–19 of pregnancy, female rats are fully maternal within one day of pup exposure. Additionally, treating nulliparous female rats with exogenous hormones for 2 weeks using a profile roughly mimicking that of mid- to late pregnancy can quickly induce mothering. This can even be accomplished in male rats! The optimal sequence of hormones that can instill mothering in laboratory rats, and probably numerous other species, is now known to involve (1) first exposing animals to estradiol, (2) subsequent introduction of progesterone, (3) administering PRL near the conclusion of the ovarian steroid treatment, and (4) the withdrawal of progesterone at the very end of treatment.[111–115]

Hormones are clearly not required for human parenting. The most salient evidence for this is that the adoption of human children in times of need, or simply by preference, yields fully parental adults obviously without

pregnancy or parturition in the adoptive parent. As discussed in the work of Fleming and her colleagues,[4,104] human parental behavior is more heavily influenced by experiential and cultural factors that parents bring to caregiving. Cultural conditions can introduce factors that are unique and in some cases more difficult to the adoptive parent–child dyad (e.g., preadoptive abuse, abandonment, or long-term institutionalization), but adoption in humans is a remarkably robust and successful process for both parents and children under a wide range of circumstances.[116–118]

Endocrine Profiles of Pregnancy Involved in the Onset of Mothering

Laboratory Rodents

Estrogen: Circulating estrogens during pregnancy in rats and mice are generally low and similar to diestrus until about 16 days after insemination, after which there is a gradual increase until parturition,[112,119,120] when plasma levels of estradiol meet and exceed those found during the estrus cycle peak.[121] Soon after parturition, there is a further brief increase during the postpartum estrus (leading to sexual receptivity ~9 h later), but circulating estrogens are otherwise low during early lactation, then slowly rise across midlactation and even more as weaning and the resumption of estrus cyclicity approaches (Figure 51.3).[122,123] In early pregnancy, the corpora lutea resulting from the liberated ova produce the steroidogenic substrates for estrogen synthesis, as well as the estradiol itself, so only estrogens are produced in large amounts.[124] This differs from late pregnancy, when the placentae produce the androgens that are released into the general circulation and from which estradiol is then produced by the corpora lutea.[125–127] During lactation, the corpora lutea of pregnancy regress, and the corpora lutea of the postpartum ovulation become functionally dominant.[123]

The presence of estradiol always induces a more rapid onset of maternal behavior than that observed in its absence.[3,4] It has a crucial role in the preparatory nest building displayed prepartum and for the immediate onset of all other maternal behaviors at parturition. A rapid onset of maternal behavior can be induced by just the first 10–13 days of pregnancy, or in nulliparous rats by 10–13 days of treatment with exogenous estradiol, if the animals are then given a triggering dose of estradiol.[89,102,128,129] In fact, depending on the females' endocrine state, even a single high dose of estradiol benzoate can shorten the latency for nulliparous rats to show maternal behavior.[129] In studies using a surgical model involving hysterectomy of pregnant females with or without ovariectomy, there is a much more rapid onset of maternal behavior when the ovary and its secretion of estradiol remain intact.[102] However, this does not occur

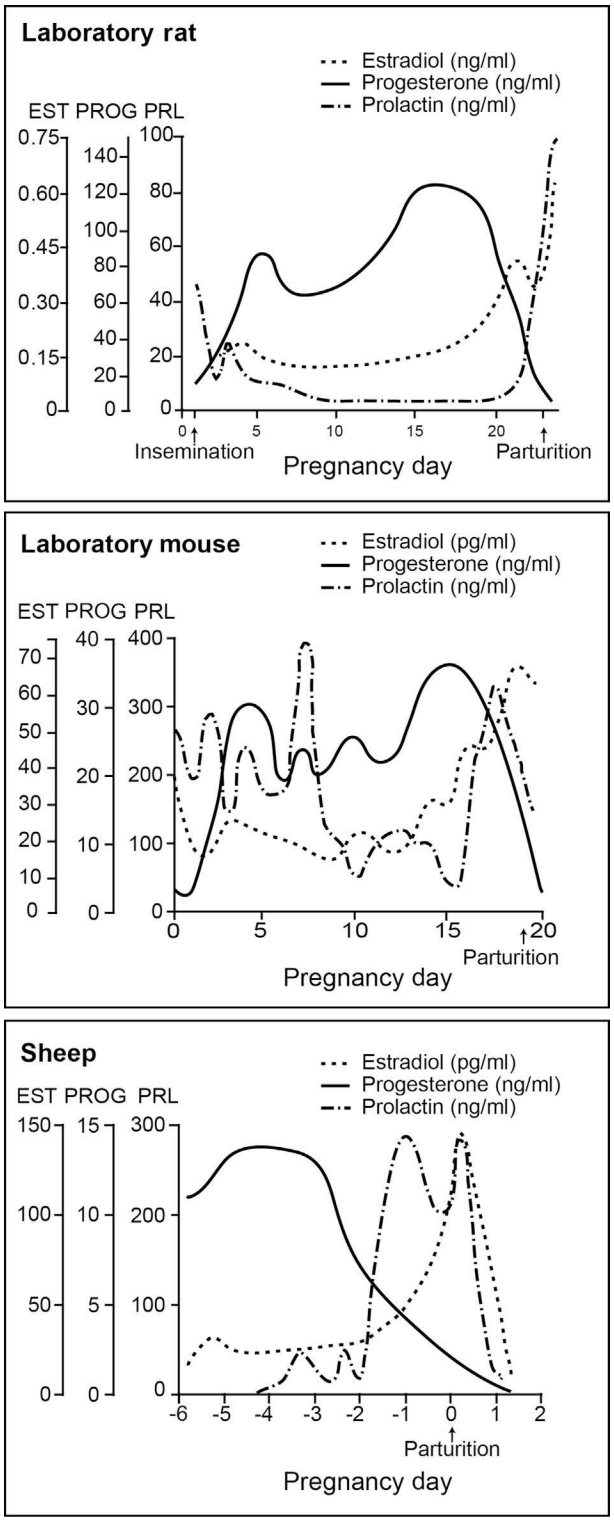


FIGURE 51.3 Schematic representation of circulating plasma levels of estradiol (EST), progesterone (PROG), and prolactin (PRL) across pregnancy and parturition in laboratory rats, laboratory mice, and sheep. *Source: Modified from Rosenblatt and Siegel, 1981.*[102]

until after day 13 of pregnancy and is sensitive to the postsurgical timing of when young are introduced.[90,130] Most of estradiol's effects on the maternal brain and behavior have long been assumed to occur genomically through activation of estrogen receptors, which have been well characterized in their distribution and density in relevant brain regions across pregnancy.[131,132] Indeed, most of the manipulations used to induce a rapid onset of maternal behavior involve a long time course of action consistent with that required for steroids to act genomically. More recently, however, Stolzenberg et al.[133] revealed the argument that estradiol also appears to act over a shorter time course, theoretically via nongenomic membrane estrogen receptors, for a rapid onset of maternal behavior.

Mice of both sexes with a lifelong and total absence of estrogen receptor alpha (ERα KO) have profoundly impaired reproductive function.[81,134] Thus, testing maternal behavior in these infertile virgin ERα KO females, which have a high rate of infanticide (40–80%),[135] is necessarily restricted to sensitization. Responding to pups appears to be reduced in ERα KO females,[81] but others have demonstrated in aromatase KO mice of a similar background strain that maternal responding does not depend on estrogens.[136] Neuron-specific conditional KO of ERα or estrogen receptor beta (ERβ) indicates that the global reproductive deficits are primarily due to the loss of ERα.[137] While global ERα KO and double ERKO (i.e., both α and β isoforms) limit the ability to study maternal behavior in naturally parturient animals, these ER knockouts have revealed valuable information about estradiol's influence on anxiety[138] and its molecular mechanisms. It had been suggested that estrogens can still act in these classical ERKO models via a membrane receptor or other novel ER-binding molecule (a possible novel ER transcript),[84] but others have found from another global ERKO model that does not contain this transcript that it has a limited role in reproduction.[134]

Although ERKO models generally have notable limits on the questions they can be used to ask, they do inform our understanding of maternal behavior in the larger context of emotional state and the neurobiological mechanisms of steroid action. The hope is that as transgenic applications develop,[139] the temporal and spatial limits of these earlier models will yield more specific information. For example, a conditional ERKO aimed only at the gonadotrophs of the anterior pituitary has been developed and resulted in subfertile females that completed pregnancy and parturition, but had smaller and fewer litters; unfortunately, no analysis of maternal behavior was included.[140] Downregulating ERα using RNA interference is another promising approach to achieve higher temporal and spatial resolution for studying estradiol's influence on mothering.[141]

Testosterone: Testosterone is an obligatory precursor to estradiol synthesis in the ovary and brain, usually in a paracrine or intracrine manner that rapidly produces estradiol, so there is usually no increase in plasma testosterone.[142] During late pregnancy, however, the rat placenta produces the androgenic substrate for synthesis of most estrogens. This results in a lesser known rise in circulating androgens (testosterone and dihydrotestosterone) from very low levels (0.4–0.8 ng/ml) early in pregnancy to sustained and remarkable levels (2.8 ng/ml) during the second half of pregnancy.[125,143] These levels are very high compared to that seen in cycling females, in which testosterone and dihydrotestosterone are found at 0.2 ng/ml to 0.4–0.5 ng/ml preceding the surge in luteinizing hormone.[127,144] These levels in late-pregnant females are even higher than those seen in male rats (1.1–2.3 ng/ml).[145] Immediately after parturition, androgen levels drop and remain low until estrus cycling recommences.

Such elevated plasma testosterone has not been reported in pregnant sheep, but high testosterone titers have been observed in pregnant mice[146] and rabbits,[147] where again the locations of estradiol synthesis and its testosterone precursor are in different tissues. While no studies have examined the effect of this androgen surge on maternal behavior in rats or mice, in rabbits it is perhaps uniquely involved in their nest building because it loosens chest hairs that females pull out and use to line the maternal nest.[148]

Androgens act on androgen receptors (ARs) located in specific tissues of the reproductive system and sites within the brain. In the brain, ARs exist in abundance in both males and females, are found on both neurons and glia, and act genomically as well as possibly nongenomically.[149,150] The AR has been a target in KO models for studying parenting and other aspects of reproduction. The first models were naturally occurring—complete androgen-insensitivity syndrome (CIAS, also known as testicular feminization mutation) in males and the XO genotype in females in which the single X chromosome had a mutated AR gene—but conditional gene-targeting approaches more recently yielded both global and tissue and cell-specific ARKO models.[86,87] One conditional model targeting the brain revealed that AR-mediated androgen action is not required for ovulation, mating, pregnancy, or lactation.[151] These females are less fertile and have fewer pups, but lactation is sufficient for normal growth of the smaller litters. No studies using these ARKO models have provided a detailed analysis of the females' maternal behavior.

Progesterone: Beginning as early as days 3–5 of pregnancy in the laboratory rat, circulating progesterone levels climb as high or up to 10-fold higher than the peak values seen during the estrus cycle and remain there for 17–18 days. Thus, a sustained high level of progesterone is one of the most remarkable features of the endocrine milieu of pregnancy. Beginning around day 19 of

pregnancy, progesterone levels markedly fall such that at parturition, they are lower than that seen at any point in the estrus cycle (Figure 51.3).[120,152,153] The temporal course of circulating progesterone in pregnant laboratory mice varies somewhat among strains, but generally peaks at days 15–17 of pregnancy and falls on the day before parturition (day 18) to half their peak levels.[146,154] On the day of parturition in rats and mice, levels fall even further after a brief postpartum estrus to very low levels, but at least in laboratory rats there is a mostly ignored elevation again between postpartum days 3 and 10, and thereafter it remains low until postweaning cycling resumes.[155]

Progesterone is the *sina qua non* for maintaining pregnancy in mammals, and withdrawal of progesterone at the end of natural pregnancy or a pregnancy-like regimen of hormones is necessary for the onset of maternal behavior. Progesterone has been demonstrated in pregnancy-terminated (hysterectomized and/or ovariectomized) and at-term pregnancy models to prevent an inappropriately early display of maternal behavior, and withdrawal of progesterone prepartum facilitates a rapid onset of mothering.[130,156] Similarly, nulliparous rats treated with exogenous progesterone require its removal for estradiol to potently stimulate mothering.[157] Evidence that progesterone at the end of pregnancy masks heightened maternal interest, rather than itself increases maternal interest simply by its withdrawal, is indicated by the fact that nulliparous rats given only progesterone followed or not by its withdrawal remain disinterested in pups.[158]

Importantly, progesterone only masks maternal behavior during late pregnancy or near the end of a pregnancy-like hormone regimen because during the early phase of estradiol exposure, progesterone synergizes with estradiol to promote maternal responding. In nulliparous rats, progesterone pretreatment reduces the dose of estradiol needed to reduce maternal sensitization latencies, and the combination of estradiol and progesterone followed by withdrawal of the latter is more effective in stimulating maternal responding than providing estradiol alone.[112,157] In laboratory mice, prepartum nest building depends on high circulating progesterone.[159] The onset of lactation also requires the presence and withdrawal of progesterone.[65,66] It was noted above that progesterone rises again beginning around the third postpartum day in rats. Early work suggested that the absence of the ovaries has no effect on postpartum maternal behavior,[160] although recently it was reported that postpartum ovariectomy slightly reduces maternal licking of pups and that nursing is somewhat impeded when circulating progesterone is experimentally elevated during early lactation.[161] The opposite result, increased nursing, is found when progesterone is elevated during later lactation, so perhaps the second decline in progesterone starting around day 10 postpartum contributes to the decline in maternal behavior in rats.[162]

The first progesterone receptor KO (PRKO) mice were designed to delete both the A and the B forms of the receptor, and both sexes sustained numerous endocrine deficits, including that females were unable to ovulate.[163] Subsequent work with isoform-specific deletions of either PR form A or B has demonstrated that PR-A KO alone leads to sterility in female mice, as well as severe uterine dysfunction, while hormonal responses of the mammary gland are mediated by PR-B.[164] Homozygous PR-B KO females are fertile, maintain pregnancy, and deliver viable, full litters of pups.[165] Unfortunately, nothing has been reported regarding the maternal behaviors of any PRKO females.

Adrenal steroids: Normal adrenal function and circulating levels of adrenal steroids optimize fertility and other needs of the female and her offspring during pregnancy, parturition, and lactation. Basal glucocorticoid levels rise in rats during late pregnancy and particularly at parturition, and thereafter return to levels more typical of early pregnancy, although there may be a flattened circadian rhythm of glucocorticoid release.[166] A notable condition of the postpartum period is that it is a time of stress hyporesponsiveness. When confronted with a variety of potential physical or psychological threats, females during mid- to late lactation exhibit a blunting of the hypothalamic–pituitary–adrenal response (see Chapter 44; and Ref. 167). An interesting feature of this hyporesponsive period is that some stressors, such as predator odor and intruders, evoke a normal stress response when the dam is in the presence of the pups,[168] suggesting a gating of the response that involves the cognitive capacity of the mother and her assessment of immediate threats to the litter.

Despite these complex alterations in glucocorticoid release in mother rats, there is no evidence that they are absolutely required for maternal behavior, as adrenalectomized females initiate and then maintain maternal behavior.[169] Detailed early studies of maternal behavior showed that the presence of the adrenal glands does facilitate the speed and completeness of caregiving in naturally pregnant females,[170,171] but others found that adrenal secretions inhibited maternal behavior.[172,173] More recent work demonstrates small but significant decreases in some components of mothering after adrenalectomy, including licking (rarely measured in early studies) and the duration of time spent in the nest, both of which could be increased by exogenous corticosterone obtained through the females' drinking water (Figure 51.4).[101] However, chronically high postpartum corticosterone via daily injections of the hormone reduces nursing,[174] and it has also been recently seen that an acute injection of the synthetic glucocorticoid, dexamethasone, impairs retrieval of pups and reduces the duration of nursing over a 30 min period following injection. Acute injection of dexamethasone also reduced peripheral PRL

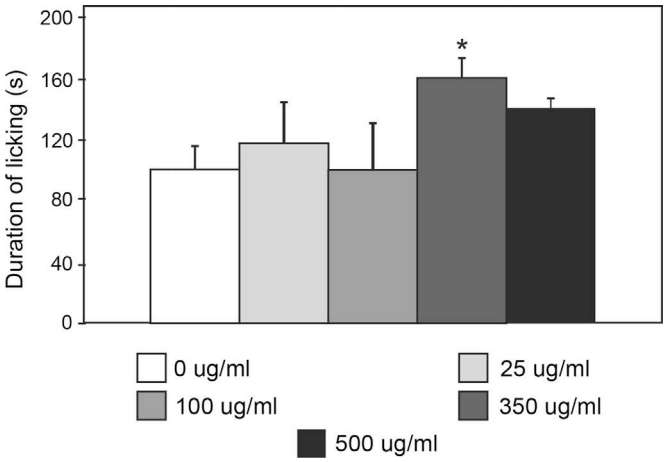


FIGURE 51.4 Duration of time (Mean + SEM) spent licking the pups by adrenalectomized, postpartum rats that received various doses of corticosterone in their drinking water. *Significantly more pup licking compared to dams that received no replacement corticosterone. *Source: Modified from Rees and Fleming, 2004.*[101]

and OT levels measured in the dams after a 15 min suckling interaction with pups, which may be a cause or consequence of the deficit in maternal behavior.[175]

In nulliparous rats, higher endogenous circulating corticosterone tends to delay maternal sensitization, and after adrenalectomy exogenous corticosterone given through drinking water or a subcutaneous pellet reduces pup licking and time spent in the nest by sensitized females that are already maternal.[176] The different consequences of adrenalectomy and/or chronic corticosterone replacement on the maternal behavior of parturient and sensitized rats clearly highlight that the endocrine and other physiological determinants of their mothering are not identical. It also appears from these studies that the effects of adrenal steroids on maternal responses may have a sphere of subtle influences that require careful consideration, including whether foster or related pups are used during testing and the postpartum stage and degree of experience of the mothers. In sum, the literature supports the view that corticosterone is not necessary for the initiation or maintenance of maternal behavior in rats, but that it modulates the intensity and timing of caregiving behaviors. It is essential to note that even such subtle influences on maternal behavior can have a significant impact on offspring development.[177]

Prolactin and its family members: PRL and three functionally related but less well-known hormones, decidual luteotrophin and placental lactogens I and II, are important during rat pregnancy because they help maintain steroidogenesis in the corpus lutea of pregnancy and prepare the mammary glands for lactation.[66] Despite their structural differences, all four protein hormones exert their effects by binding to the short or long form of the PRL receptor. Both forms of the PRL receptors are expressed and regulated differentially across reproductive states in the corpus lutea, mammary gland, and brain (see Chapter 12; and Refs 178–182).

The source of PRL itself during the estrous cycle and during early pregnancy is the anterior pituitary. During early pregnancy, PRL is secreted in two daily surges, one nocturnal and one diurnal.[183,184] At the same time, decidual luteotrophin is secreted by the decidua of the uterus, so PRL is thereafter no longer required to maintain pregnancy even though the anterior pituitary continues to release it.[181] Subsequently, in midpregnancy serum PRL from pituitary origin is substantially lower than what is found in cycling females, and decidual luteotrophin disappears while placental lactogens, first I and then II, become the predominant lactogens in the general circulation.[120] During the second half of pregnancy in rats, pituitary PRL secretion is inhibited by placental lactogens through negative feedback on the brain, but this is alleviated in the last few days of pregnancy. The pituitary then releases a substantial prepartum surge of PRL (Figure 51.3).

During the postpartum period, PRL has a critical role on the mammary gland for milk production and in the corpus lutea for progesterone synthesis.[123] Of course, suckling stimulation by pups is a powerful regulator of PRL release, such that there is a sharp increase especially when a female rat is separated from and then reunited with her pups. This is observed even after transecting the galactophores (milk ducts), which leaves the sensory capacity of the nipples intact but prevents suckling-induced milk letdown.[185] Maternally sensitized nulliparae do not respond to the presence of pups with an elevation in PRL, though.[186] Across the first 4 days after parturition, PRL levels rise such that they are 2–3 times higher than the maximum of the estrous cycle and remain so during the first 10 days of lactation, after which they decline quickly and remain relatively low until the end of the postpartum period and when estrus cycling resumes.[121,123,183]

PRL greatly facilitates the onset of maternal behavior that is initiated by ovarian hormones. Systemic injection of PRL included in a regimen of exogenous estradiol and progesterone, followed by withdrawal of the latter, notably reduces maternal sensitization latencies.[111,112,187] Moreover, Bridges and colleagues demonstrated that administering these ovarian hormones cannot decrease maternal sensitization latencies in ovariectomized female rats if they have also been hypophysectomized, and that ectopic pituitary grafts can reinstate the hormones' effects.[188] Even so, late-term hypophysectomy does not interfere with parturition or the onset of maternal behavior, which is presumably because by that time placental lactogens have assumed the major role in providing PRL to the maternal brain and periphery.

Access of PRL to the brain occurs via an active transport mechanism, possibly related to the abundant PRL receptors in the choroid plexus.[189] Grattan and colleagues

report that there may also be differential sensitivity, and possibly increased brain access, of PRL to some hypothalamic regions involved in the feedback regulation of PRL by dopaminergic neurons of the arcuate nucleus.[190] There is also evidence that PRL is synthesized by intracerebrally projecting neurons within the brain and in the neurons of somatosensory ganglia, and that it can be released site specifically in the female rat brain independent of peripheral release.[78,191]

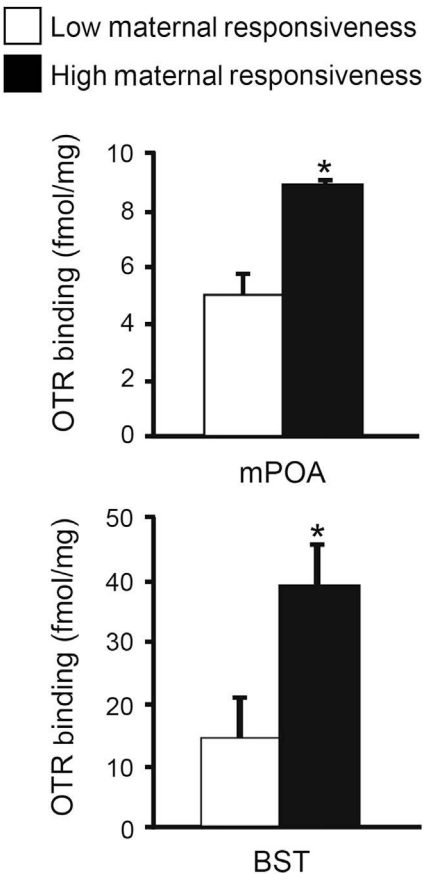
In laboratory mice, postpartum maternal nest building is facilitated by PRL,[159] but estradiol-mediated retrieval in ovariectomized mice that do not already spontaneously retrieve is unaffected by PRL depletion.[192] Mouse models exploring the role of PRL in maternal behavior have included the use of complete knockouts of either PRL or its receptors. As reviewed in depth by Kuroda et al.,[81] the earliest studies of PRL KO mice showed that the maternal responding of the virgin females is normal. Either another PRL-like ligand must be engaged in a compensatory mechanism in the adults or PRL is not absolutely required for maternal behavior in the virgin laboratory mouse. An ontological consideration is that PRL family ligands may crucially act during fetal development in brain regions needed for maternal behavior and that this remains intact because the numerous ligands are not eliminated by the PRL KO alone. These limitations highlight the importance of choosing background strains of mice for KO that are not spontaneously maternal as virgins, and so might not require the hormone in question in the first place. In contrast to eliminating the gene coding for PRL, KO of the PRL receptor gene does diminish maternal behavior.[193] Thus, it has been speculated that there is a role for PRL in these mice that can be filled by other ligands, possibly the placental lactogens acting during pregnancy in subjects with normal PRL receptors, but not in the absence of the PRL receptors.[4]

Oxytocin: OT is released during the beginning of pregnancy as a result of mating stimuli, and it may participate in the twice-daily PRL surges that maintain the corpus lutea necessary for steroid secretion,[85] but any relevance for this early OT in later maternal behavior is unknown. Preparturitional restraint of peripheral OT secretion is important to avoid premature contractions of the uterus or milk ducts, but during pregnancy OT does rise up to twofold over that observed in nonpregnant males and female rats and humans, with a peak at parturition and later peaks during suckling in all species examined.[194,195] During pregnancy, there are modifications of the hypothalamic magnocellular system involving retraction of astrocytic processes separating OT cells, thereby allowing more synchronous firing when a bolus of OT is needed for peripheral processes, including parturition or milk letdown (see Chapter 13; and Refs 196,197).

There is considerable evidence that OT can promote the onset of maternal behavior but it is not required for maintenance of the behavior once established. Prepartum destruction of the hypothalamic sites containing OT cells impairs all aspects of mothering after parturition, but lesions performed postpartum have only small effects.[198,199] OT from peripheral plasma is probably not involved because its access to the brain is extremely limited.[76] Some have argued that peripherally secreted OT as measured in plasma predicts release within the central nervous system,[200,201] but there can be brain-site-specific OT release and action independent of plasma levels.[79,202] The best evidence clearly indicates that OT facilitates maternal behavior by acting as a neuromodulator within the brain, and is released from a well-described network of neurons sometimes in association with other events, including parturition and nursing.[80,203,204]

Evidence of OT's modulatory role in postpartum maternal caregiving has been established by classical approaches, including infusing it into the brain or, conversely, infusing OT antiserum or receptor antagonists into the cerebral ventricles or relevant brain regions. These studies in rats involve postpartum females, pregnancy-terminated females, and pup-sensitized virgins.[108,204–207] There are also positive relationships between facets of the brain's OT systems with the propensity to display some postpartum maternal behaviors, including kyphosis and licking of pups (Figure 51.5).[108,208] Early studies of the role of OT in maternal behavior sometimes compared it with the effects of the closely related "control" neuropeptide, AVP. In contrast to these early works, where the effects of AVP were explained by its actions on the OT system, more recent research indicates that AVP acting on its own receptors also regulates maternal behaviors toward pups and maternal aggression.[204]

Transgenic mice have revealed a tremendous, often surprising, body of evidence about OT's modulatory role in maternal behavior. Mice with total global KO of the gene coding for OT give birth normally but have marked deficits in milk letdown. No deficits in maternal behavior were initially reported in these KO mice,[209] but more detailed analysis of OT KO mice subsequently revealed deficits in both retrieval and licking.[210] Because maternal behaviors are less affected in these OT KO mice than probably was expected, compensation by other systems (e.g., AVP) may have occurred or the strain used to create these particular KO mice has a high baseline level of spontaneous maternal behavior that was only slightly blunted.[209] In fact, it is valuable to note that in female wild house mice that are infanticidal as virgins, intracerebral infusion of OT dramatically promotes mothering.[211] Knockout of the *CD38* gene that codes for a protein critical for calcium-induced OT release does not impair the fertility, pregnancy, or parturition, and curiously also does not affect lactation in laboratory mice. This may be explained by the fact that while these mice have reduced circulating OT, it is sufficient for milk letdown.



FIGURES 51.5 **[^{125}I]OTA binding in the mPOA and BSTv (adjacent brain sites critical for active maternal behaviors; see the section Hormones Most Significant for the Onset of Maternal Behavior) of postpartum female rats that had previously displayed high or low maternal responsivity (i.e., high or low licking and nursing).** Highly responsive females had greater [^{125}I]OTA binding in both brain sites compared to less responsive females. *Source: Modified from Champagne et al., 2001.*[108]

This *CD38* KO does produce behavioral deficits, including slower retrieval, disorganized retrieval, and slower return to nursing after a disruption,[212] suggesting that brain OT release in these animals is insufficient to maintain normal mothering.

The most specific action of OT in the brain and periphery is via a single isoform of the OT receptor, which increases in number as pregnancy progresses and at parturition.[213] A total KO of the OT receptor in the brain and periphery revealed that parturition is surprisingly normal, but that lactation is not possible, and some aspects of postpartum or spontaneous virgin maternal behavior are reduced but not absent.[214] The maternal behavior deficits in this OT receptor KO are also probably more subtle than expected, again suggesting compensatory responses by other systems. Using a conditional OT receptor KO confined to the forebrain, and hence sparing the peripheral receptors and their function, others reported no impairment in maternal behavior, although pup licking was not assessed.[213] While these mice have apparently normal lactation, they are plagued by pup mortality of unknown etiology. The surprising retention of maternal function of these OT receptor KO mice may depend upon residual OT receptors within the forebrain, or possibly compensation from other brain regions outside the targeted brain region. An example of such compensatory mechanisms comes from the periphery and is related to OT's luteotrophic effect, such that prostaglandins take over this function in the absence of the OT receptor, calling attention to the fact that compensatory responses in the KO models may be more wide ranging than is currently proposed in many studies.

Sheep

Estrogen and progesterone: Virgin ewes are either indifferent or aggressive toward lambs, and, unlike rodents, they remain so even if they have prolonged exposure to young. This is radically changed by pregnancy, parturition, and a brief interaction with the offspring postpartum. Levels of estrogens are low during pregnancy and rise only very late and briefly in the peripartum period to a level approximately five times that seen at the peak of the estrus cycle. They then fall within hours after delivery.[102,215,216] Circulating progesterone levels are also very low in early pregnancy but rise later in pregnancy and are sustained at approximately four times the peak amounts found during the estrus cycle.[102,216] As pregnancy progresses in sheep, the source of progesterone shifts from the ovary to the placenta.[217] The progesterone rise as pregnancy progresses in ewes is similar to what is found in rats, such that levels are high until they rapidly fall a few days before parturition, and then remain at low levels through the early postpartum period (Figure 51.3).[102,215]

The high estradiol and low progesterone during very late pregnancy and the early postpartum period are positively related to maternal responsiveness in ewes, including lamb grooming, acceptance of suckling attempts, and maternal vocalizations by the ewe.[102,215] Maternally experienced ewes show increased responding to newborn lambs about 10 days before parturition,[218] presumably facilitated by the change in circulating hormones during late pregnancy. The hormones of late pregnancy also mediate the process where sheep withdraw from their herd before parturition, and give birth to and bond with their offspring in relative isolation. This isolation behavior is considered one of the earliest signs of maternal responsiveness in the pregnant ewe.[219] Fifty to sixty percent of multiparous ewes can be stimulated into maternal acceptance using 7–30 days of exogenous estradiol and progesterone treatment, with the best acceptance found with regimens involving high estradiol and low progesterone.[219–221] Unlike rodents, physiological levels of estrogens alone are insufficient to stimulate complete maternal behavior in sheep, but

together with the sensory input and OT release during vaginocervical stimulation (which is also insufficient alone), most primiparous ewes will completely and rapidly accept lambs.[96,221–223]

During the postpartum period, there is a hormonally established window of sensitivity to offspring during which the ewe must physically interact with the lamb and display placentophagia in order for them to form a preference for their own lambs. These activities have to occur within 4–12 h after parturition, and their effects usually begin to wane within 6 h of parturition.[218,224] The hormonal and visceral somatosensory stimuli, the chemosensory stimulation from the newborn lamb and amniotic fluids, and the auditory stimuli provided by low-pitched "lambing" bleats from the ewe provide the basis for bonding between the ewe and her offspring.

Adrenal steroids: Plasma cortisol levels are elevated during late gestation and lactation in ewes.[220] Cortisol levels during parturition are negatively associated with the onset of affiliative behaviors exhibited by the ewe to the lamb, but breeds that are more maternally responsive have higher levels of cortisol during the postpartum period.[220] It remains to be seen whether these higher levels simply do not interfere with maternal attentiveness, or possibly promote it, as has been suggested for humans (discussed further in this chapter). In support, hypothalamic–pituitary–adrenal (HPA) axis stimulation in sheep, along with additional hormonal treatments, can facilitate maternal acceptance by nulliparous ewes.[4,220] There is also evidence that the induction of parturition with exogenous dexamethasone shortens the sensitive period for bonding with the lamb due to reduced postpartum estrogens, which normally maintain this sensitive period.[218,224]

Prolactin and its family members: Similar to other species, basal PRL is low during most of pregnancy in sheep, but there is a substantial increase in the days immediately before parturition to reach levels somewhat higher than those seen in cycling females.[102,225] Placental lactogens also follow a similar pattern to that seen in other species, with sustained levels during later pregnancy and higher levels when two or more fetuses are being gestated.[226] An important difference between the scientific literature on rodents and ewes is that there is little evidence that PRL is required for the ewe's maternal behavior or bonding with the lamb.[4,219]

Oxytocin: Plasma OT levels peak at parturition in ewes, then fall to levels similar to prepartum levels within 24 h after parturition. They remain low during the postpartum period except for peaks associated with suckling by the lamb.[220] Plasma OT does not vary across breeds of sheep with higher versus lower maternal care, and peripheral OT is not thought to be relevant for the onset or maintenance of their maternal behavior.[220] Instead, Kendrick and colleagues have used microdialysis to assess OT release and infusion of OT to demonstrate that the location of OT action to promote mothering is intracerebral, particularly the olfactory bulb and paraventricular hypothalamus (PVN).[95,202,227] These investigators and others have also demonstrated that opioids acting within the brain facilitate maternal responsiveness, including regulating OT release, which is dissimilar to the disruptive effects of opioids on mothering in laboratory rats (see the section Brain Control of Maternal Behaviors).[228]

Humans

Due to the dramatic expansion of the cerebral cortex that occurred during primate evolution, mothering by humans is thought to be more cognitively based and greatly emancipated from the hormonal events often necessary for the stereotypical and reflexive caregiving behaviors displayed by many other animals.[229] Studying the hormonal basis of maternal or parental behavior in nonhuman and human primates is, of course, much more difficult than studying the nonprimate models described in this chapter. Some very significant work has been possible in nonhuman primates, though, and demonstrates roles for estrogens, cortisol, PRL, and OT that echo information found in nonprimates as well as some findings in humans.[230] The greatest limits to studying this question in humans is that most experiments can only investigate circulating levels of hormones assayed in plasma or saliva and correlate those measures to naturally occurring variance in behavior. An additional difficulty is the complex and highly varied nature of human maternal responsiveness, which limits generating an operational definition of maternal behavior that is relatively simple in animals with more stereotyped behaviors. Nonetheless, some laboratories have created useful observer-based operational scales that are appropriate for defining features of human mothering and they have correlated those measures with hormone levels.[231,232] The complicated issues introduced by our emotional states and traits affecting our behavior have usually been addressed by statistically considering data obtained by self-report or clinically administered psychological scales examining state (current affective state, postpartum depression, and anxiety) or trait features of our personalities (depression across life, personality, and pathology).[233]

Given the demonstration from animal models that hormones are important for the initiation of maternal behavior, many studies of maternal behavior in humans pay close attention to hormone levels during pregnancy and soon after giving birth. Indeed, this strategy has provided the best clues about possible hormonal influences in humans.[231,234] While the role of hormones in human mothers is mostly without the causal proof available

from studies of nonhumans, the natural endocrine patterns during this period have been well documented, which is essential for uncovering any role for hormones in human mothering.

Estrogen, testosterone, and progesterone: As with other mammals, the human placenta and the fetus contribute to the endocrine state of the mother, particularly during the late second and third trimesters, so delivery results in a rapid reduction in many circulating hormones within 24–48 h. Humans and nonhuman primates have been consistently reported to have a temporal course of estrogens and progesterone during pregnancy that is similar, but not identical, to those described in the rat and sheep models described here. Estrogens rise more steadily across pregnancy in humans so that in the second and third trimesters, plasma levels are 10–100 times those seen at the highest points of the menstrual cycle (e.g., the midluteal phase).[75,235–238] These levels remain high at delivery but fall substantially by the third postpartum day, such that they are below menstrual cycle levels by day 14 postpartum and remain low for up to 12 weeks postpartum in lactating women.[238,239] In nonbreastfeeding women, the endocrine changes consistent with the resumption of cyclicity begin in the first month postpartum (Figure 51.6).[240]

Less frequently analyzed or discussed for human pregnancy and the peripartum period is plasma testosterone. Similar to the rat, human circulating testosterone during the third trimester is three times the level found at the highest point in cycling women (i.e., midluteal).[238] While concentrations of testosterone are substantially less than those of estradiol during pregnancy (3 versus 82 nm/l), it remains at these levels for much longer than that occurring during the menstrual cycle, and so is a unique feature of the endocrine milieu of pregnancy.

Progesterone levels rise steadily across human pregnancy such that in the second and third trimesters, they are about 100 times higher than the levels found during the follicular phase of the menstrual cycle and 6–50 times higher than the maximal levels of the luteal phase of the cycle.[75,237,238] In terms of relative concentration, progesterone is the dominant gonadal steroid hormone in human pregnancy, being 10-fold that of even the substantially elevated estrogens.[237,238] Until late pregnancy, progesterone levels follow a pattern similar to that discussed here for other animal models.[235,236] Unlike other placental mammals such as rats, mice, and rabbits that have precipitous drops in plasma progesterone just before parturition, human circulating progesterone does not drop so substantially until after the fetus and placenta are expelled. In fact, only on the first day postpartum does progesterone fall precipitously to levels similar to what is found during the luteal phase of the cycle.[239,241–243]

The absence of a dramatic withdrawal of circulating progesterone before parturition in humans is intriguing because when labor begins, there is both high circulating progesterone and the formidable onset of myometrial contractility. There is thought to be a functional "progesterone block" that is alleviated prior to the onset of labor when the myometrial contractions are otherwise held in check by progesterone.[244] Mechanisms for how progesterone's function in the uterus is blocked during labor include a change in the dominant form of the genomic progesterone receptor, from the B to the A form, and subsequent release of myometrial contractility.[245–247] Interestingly, nongenomic progesterone receptor activity may favor myometrial contractility rather than inhibit it.[244]

Adrenal steroids: Primarily due to the contributions by the fetus and placenta, cortisol levels rise substantially across human pregnancy, rising 30% in the first trimester and by nearly threefold in the last two trimesters.[237,238,243] In the last trimester, plasma cortisol is nearly double that found in nonpregnant women.[75] In a within-subject longitudinal study, it was found that cortisol fell to slightly higher than prepregnancy levels within 2 days postpartum,[248] resulting in about half that seen during late pregnancy and labor,[243] and that the levels remained there for at least part of the postpartum period.[249] Walker and coworkers recently showed that the daily rhythm of cortisol returns to its normal circadian course and to nonpregnant levels in weeks 5–20 postpartum and that these

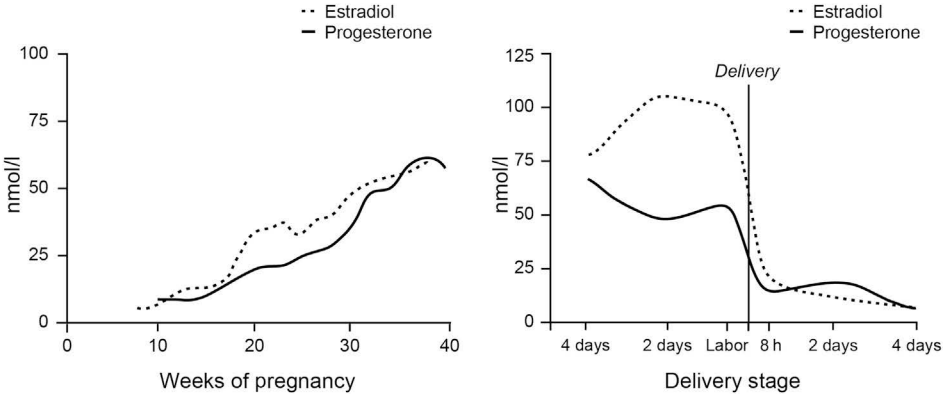


FIGURE 51.6 Schematic representation of circulating plasma levels of estradiol and progesterone across pregnancy and parturition in humans. Note that the levels of progesterone shown are divided by a factor of 10. For example, the immediate prepartum progesterone level is actually approximately 500 nmol/l. *Source: Modified from Brett and Baxendale, 2001.*[749]

levels are not altered by the mother's parity or whether she breastfeeds or bottle feeds the infant.[250,251]

Prolactin and its family members: Plasma PRL levels rise about 10-fold during pregnancy, without significant changes in cerebrospinal fluid levels.[75,238] As in other mammals, placental lactogens rise across pregnancy in humans to levels much higher than those of PRL itself. There have been no measures of PRL levels in the brain itself, but because PRL transporters are present in the choroid plexus, the human maternal brain is probably chronically exposed to high amounts of all PRL-type hormones from the periphery and may also be affected by PRL of purely central origin. The structurally similar placental growth hormone is also present at high levels in maternal blood, but less is understood about its access to her brain.[252] Placental lactogen is involved in lactogenesis and steroidogenesis, but human pregnancy is fine without it, possibly due to compensation by the structurally similar growth hormone.[252,253] However, data from mice deficient in growth hormone that are also lactogen resistant suggest that these hormones have distinct functions.[254] Within a day after parturition in women, placental hormones disappear from the blood stream and PRL itself is thereafter the dominant hormone supporting lactation.[236] Because PRL has a role in onset of maternal behavior in most animals examined, it seems possible that this is true for humans, but it has not been established in correlational studies. Whether such PRL effects would depend on peripheral PRL acting on the brain, or by PRL both made and acting within the brain, is a viable question.

Correlations among circulating hormones and human maternal behavior: There is a positive relationship between the ratio of the levels of estrogens:progesterone in human mothers and higher levels of feelings of attachment in the peripartum period, as well as increased approach and appropriate contingent responsiveness to infant cues, further providing evidence that these hormones in nonhuman primate females are related to behaviors thought to typify a good mother.[4,104,230,255] There is also work from psychologists and anthropologists supporting a role for circulating estrogens for maternal tendencies (i.e., interest in and desire to have children) and other features often associated with feminization.[256]

As discussed in detail in the section Hormones Most Significant for Paternal Behaviors, the first findings concerning testosterone and parental behavior were that lower circulating testosterone correlates with higher responsiveness in human fathers. A similar correlation has also been reported in women, with lower testosterone levels associated with motherhood and long-term social attachment such as that found in marriage,[257,258] whereas lower scores on maternal interest and reproductive ambition were found in women with higher circulating testosterone.[259] Circulating testosterone was not correlated with general career ambition, suggesting a restricted realm of influence for testosterone in women.

Novel work from Fleming and colleagues demonstrates that, among the steroid hormones, adrenal steroids are the most highly and positively correlated to high maternal responsiveness in women.[104,232] Specifically, mothers with higher levels of circulating cortisol interact more sensitively with their infants,[260] are more sympathetic to cries,[261] and are more attracted to and can better discriminate among infant odors (Figure 51.7).[232,261] This may indicate that higher cortisol contributes to a level of arousal that is optimal for maternal responsiveness, although overarousal would presumably interfere with appropriate mothering. These data were obtained on a single afternoon (a circadian point of decreasing levels) in a hospital setting, which may itself increase basal cortisol, 2 days after delivery when cortisol would be expected to have fallen to a point somewhat above prepregnancy levels. In contrast to these results, Feldman et al. reported that higher cortisol levels were associated with lower maternal behavior in a population of both primiparous and multiparous women of diverse socioeconomic status.[231] These findings were based on repeated measures taken in a clinical setting during the first and second trimesters and then 4 weeks postpartum (the time of day of cortisol sampling was not specified).

Lactating women, like other mammals, have attenuated HPA axis responses to stressors.[262] While some have reported reduced HPA axis responses to a social stressor immediately after nursing compared to women who simply held their infants,[263,264] others find this only in multiparous women[250] or if the time between feeding the

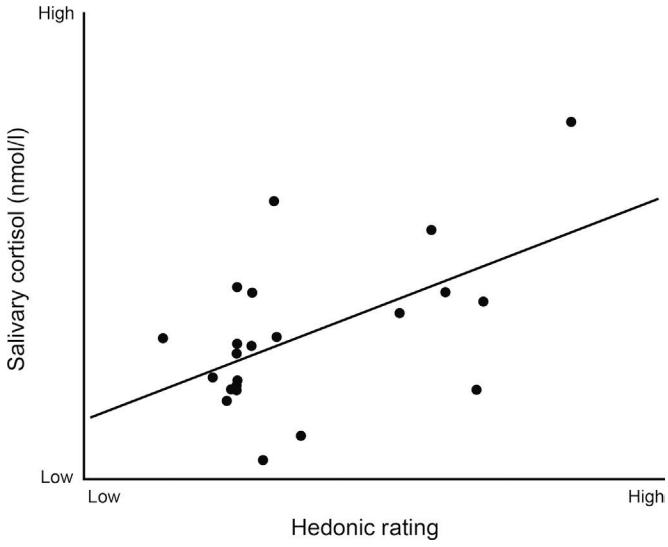


FIGURE 51.7 Correlation between salivary cortisol and the hedonic ratings (i.e., how pleasant or unpleasant) made by first-time mothers about the odor from their own infant's shirt. Mothers with higher cortisol rated the odors as more pleasant. *Source: Modified from Fleming et al., 1997.*[232]

infant and exposure to the stressor was relatively long.[265] Furthermore, suckling by the infant exerts a shorter term restraint on the HPA axis in humans compared to the longer term effect lasting even a day or two after litter contact seen in laboratory rats.[263]

Differences among studies in the relationship between adrenal steroids and mothering or stress responsiveness in parturient women highlight how sensitive such results are to a myriad of factors. This includes when the hormones are measured, which is particularly relevant for adrenal steroids that show marked circadian rhythm and very dramatic changes across pregnancy, parturition, and the postpartum period. Further influences include socioeconomic status (which is inversely correlated with basal cortisol), cultural and social conditions, infant feeding choices, and parity.[250,251] Also relevant is the potential for personality trait dependence of such findings, possibly including those underlying variations in basal and reactive cortisol responses.[104,249] In any case, while the bases of the discrepant findings are unknown, they are worthy of further exploration.

Among the peptides, OT has been the most studied for a relationship with human maternal care. OT released into the peripheral circulation has well-known roles for uterine contractions at parturition and postpartum milk letdown, with plasma levels of OT elevated during late pregnancy and in response to postpartum suckling.[75,266] While a few findings suggest that peripherally available OT has the potential to alter human maternal behavior, these studies do not provide any mechanism by which these effects occur as OT cannot cross into the brain. Intranasally administered OT does substantially raise salivary OT levels, with a short-lived peak after administration, but more work needs to be done in humans to determine the relationship between naturally occurring brain and peripheral OT.[267]

Without being able to define whether brain or peripheral OT is involved, there appears to be a role for the extended OTergic system in maternal attachment and care of infants. Circulating OT levels across pregnancy and the postpartum period are higher in women who are the most attached to their infants, and these women also exhibit higher levels of some maternal behaviors.[231] Additionally, mothers who are highly affectionate with their infants have higher circulating OT following an interaction with them compared to women rated as providing low levels of affectionate contact.[200] Polymorphisms in the genes coding for the OT receptor or CD38 are also associated with mother–infant bonding,[268] a finding reminiscent of the relationship between variation in OT receptor expression and some maternal behaviors in laboratory rats.[208] Furthermore, 6–14-week postpartum mothers with the greatest increase in plasma OT in response to a play interaction with their infants score highest on orienting sensitivity and attention to the mood, sensory cues, and emotions of their infants. Conversely, women who scored highest in trait measures of effortful control and focus on executive plans have lower levels of OT.[233] These interesting results suggest that the quality of certain mother–infant interactions are related to OT even during the later postpartum period, and that stable personality traits may predict some of these interaction qualities and their relationship with OT release.

HORMONES MOST SIGNIFICANT FOR PATERNAL BEHAVIORS

Paternal care is found across a large swath of the animal kingdom, from insects to humans. Birds are the most paternal vertebrates, with over 90% of species displaying biparental care, and the endocrinological basis of caregiving in avian fathers is quite well studied.[269,270] Fathering in mammals is much rarer, seen in only approximately 5–10% of species,[1,271] and is most commonly found in rodents, canines, and about 40% of primates. Laboratory studies of male rodents, such as laboratory rats, that are not parental in their natural environments reveal that the behavior can still be induced after manipulations that include early feminization by neonatal castration, treatment with very high doses of ovarian hormones during adulthood, and/or prolonged exposures to neonates.[2,28] Thus, the conclusion can be made that many male brains contain the necessary substrates for expressing paternal behaviors, regardless of natural history, and that this latent system can be uncovered under precise laboratory conditions.

Natural paternal involvement in caregiving is often coupled with a monogamous mating system and its associated increase in, but not absolute certainty of, paternity. Paternal involvement is also more likely expressed in relatively harsh environments in which food resources are sparse or temperatures are extreme, such that caregiving from two parents can greatly contribute to offspring survival and reproductive success.[1,271] Fathers of biparental species can also provide additional protection and supervision of the offspring. Studies of the benefits of fathering within a laboratory setting probably underestimate its importance given the undemanding environment,[28] although litter growth and survival have still been observed to be compromised in the absence of a sire even within the laboratory.[272–275] In humans, many studies have shown that the absence of a biological father or a second highly invested caregiver can contribute to negative social, psychological, and physical health consequences for children.[276]

We here review the endocrinology of paternal behavior in some exemplar parental mammals, with a focus on species displaying obligate paternal behavior within their natural environments, meaning that paternal care is

continually displayed by most sires of the species. This can be contrasted to the facultative fathering that is expressed more sporadically by sires in other species only during times of particular need and when mothering alone is inadequate.[28] For example, with approaching winter temperatures, many typically nonpaternal meadow voles (*Microtus pennsylvanicus*) nest with females and interact positively with their pups.[277] Such facultative fathering is not very often studied endocrinologically or neurobiologically, but is particularly intriguing because it requires the behavior to be switched on and off, sometimes repeatedly in the same individual across their lifespan. These transient plastic changes in the brain necessary for facultative fathering may involve the same systems necessary for paternal behavior in obligate fathers.[278] It is also interesting to consider that the behaviors of these more flexible facultative fathers are more likely susceptible to environmental influences compared to the relatively hard-wired behavior of obligate fathers.[279]

Studies of the endocrinology of fathering have often followed the heuristic that changes in steroid and peptide hormones that compel males to act paternally are similar to those necessary for females to display the same behaviors (i.e., elevated E, PRL, and OT). It will be seen that this assumption is not always correct and that the hormonal profiles associated with fathering considerably differ across species, especially among rodents.[28]

California Mice

The California mouse, *P. californicus*, is an important model system for investigating the hormonal control of paternal behavior. In contrast to most other socially monogamous species, including prairie voles,[280] DNA fingerprinting and paternity analysis suggest that wild California mice have extremely low rates of extra-pair fertilizations and are essentially strictly monogamous.[281] Equivalent levels of caregiving behaviors are expressed by males and females, with the obvious exception of nursing,[282,283] and offspring survival relies tremendously on the care provided by their father.[284] Virgin male California mice are not particularly attracted to pups and often attack them, but they become more paternal in response to olfactory cues they receive while living with their pregnant mate and are very paternal after the pups are born.[2] The hormones and neuropeptides studied for paternal behavior in California mice are numerous, with testosterone and its intraneuronal aromatization to estradiol receiving the greatest attention.

There is a classical inverse relationship between circulating testosterone and parenting that was first established in paternal birds[285] and that alters males' time allocation to other reproductive endeavors, including aggression, attracting mates, mate guarding, and an increased focus on their mate.[286] Further studies reveal plasticity in this hormone–behavior relationship, particularly when there is temporal overlap among mating, aggression, and paternal behaviors.[282,287] For example, California mouse pairs mate during the postpartum estrus, but males need to coordinate this with mate guarding and behavior toward their young pups in the nest. Behavioral conflicts do arise, as in one case observed in which a male California mouse removed the pups from the nipples of his female mate and placed them in the corners of the cage prior to mating with her (Marler, unpublished data).

As predicted from this classical perspective, testosterone levels in male *P. californicus* peak at courtship and significantly drop when they become fathers (Figure 51.8).[288] However, there is a *positive* correlation between testosterone and huddling with and grooming of pups in experienced fathers, and this relationship is not seen in virgin males.[289] A similar positive relationship between circulating testosterone and huddling with young pups has been found in another biparental male rodent, the Mexican volcano mouse (*Neotomodon alstoni*).[290] This positive hormone–behavior association is further supported in California mice by the detrimental effects of castration on their paternal care and the resumption of fathering after reintroduction of testosterone.[291,292] At first glance, it seems difficult to assimilate these seemingly contradictory findings, but there is an interesting evolutionary twist in male California mice because testosterone influences paternal behavior through activity of estrogen receptors rather than androgen receptors. Testosterone is aromatized into estradiol locally in specific brain regions to influence paternal behavior in California mice, as first suggested by the finding that implants of estradiol are as

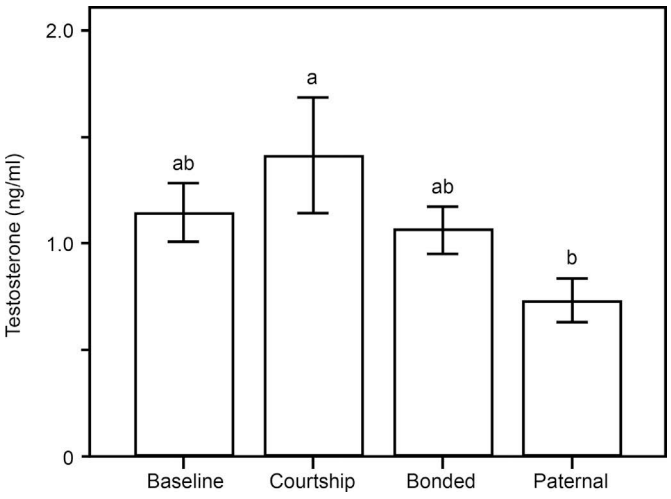


FIGURE 51.8 Circulating testosterone (Mean +/- SEM) in male California mice before pairing with a female (Baseline), an hour after pairing (Courtship), 3 weeks after pairing (Bonded), and 4 days after the birth of their pups (Paternal). *Source: Modified from Gleason and Marler, 2010.*[288]

effective as testosterone in maintaining paternal behavior in castrated males.[292] In addition, aromatase activity in the medial preoptic area (mPOA), a brain area critical for expression of most parental behaviors in all species studied (see the section Sensory Control of Maternal Care), is significantly elevated in fathers compared to mated males without pups.[293] Evolutionarily, it is possible that *P. californicus* males have successfully co-opted some of the mechanisms used in maternal care while avoiding some of the high costs of testosterone.

The relationships between glucocorticoids and the physiology and behavior of rodent mothers were reviewed above in the section Hormones Most Significant for the Onset of Maternal Behavior, but studies of male California mice indicate they may be mostly resistant to such glucocorticoid effects. This is suggested at a physiological level by their very high basal corticosterone levels, the inability of even moderate doses of dexamethasone to suppress this endogenous release, and the fact that several stressors are unable to further raise corticosterone levels but instead lower them at the time of the high diurnal peak.[294] Nonetheless, males' corticosterone does increase in response to a predator odor at times during the day when corticosterone levels are naturally lower,[294] as in response to social defeat at any time of day.[295] With regard to a relationship between glucocorticoids and paternal behavior, the published data are conflicting. A single injection of corticosterone administered in a manner that mimics an acute stress response has no effect on males' paternal behavior.[296] However, a complex analysis that included corticosterone, dehydroepiandrosterone (DHEA), and interruption of behavioral patterning in response to exposure to a novel object found greater levels of circulating corticosterone and DHEA but less behavioral disruption in fathers compared to sexually inexperienced males.[297] Others have found no difference in basal circulating corticosterone among males assessed as virgins, mated but without pups, and fathers.[298] In a study examining immediate-early gene activity of cells in the hypothalamic PVN that produce corticotropin-releasing hormone (CRH), there was no difference between paternal and nonpaternal males in response to stress.[299] Overall, male California mice appear to be resistant to stressors through both behavioral and HPA axis changes. What is unclear is whether this relates to the transition from being sexually inexperienced to becoming a father. Even if it does not, there may have been general selection for these males to respond less to stress because of the monogamous nature of their mating system. Males that mate for life and that have few offspring per litter are predicted to have buffers against stressors, unless a stressor strongly impacts the survival of the family unit or their ability to mate guard. Whether this also involves a change in emotional state in California mice is unclear because there are variable results regarding the effects of fatherhood on anxiety, fear, and neophobic behaviors,[297,299,300] even though fatherhood does appear to suppress activity in brain areas underlying these emotional behaviors.[301]

Correlational analyses of other plasma hormones in male California mice indicate little evidence that OT is important for paternal behavior. OT levels are high in males the day following mating, but then remain low throughout the mate's pregnancy and later development of the offspring.[302] More compelling are the findings that PRL rises within 2 days after the birth of a litter[303] and that progesterone levels drop with the onset of paternal behavior.[293] Exogenous PRL has been seen to rapidly induce paternal responding even in the typically nonpaternal male laboratory rat,[304] but additional studies are needed to assess cause-and-effect relationships between PRL or progesterone and paternal behavior in *P. californicus*.

Wild House Mice

Feral wild house mice live in extended social groups that often consist of an adult breeding male, several females and their litters, and a number of subordinate males.[305] Similar to other rodents living under such conditions, the expression of paternal behavior is likely in their natural environment. In the laboratory environment, almost all virgin males are infanticidal but can be parentally sensitized, and after mating they will refrain from killing pups when tested early or late during their mate's pregnancy.[82,306] The stimuli capable of inhibiting infanticide in mated wild male house mice are numerous, and this inhibition can occur from copulation alone[82] or by cohabitation with a pregnant female.[306] Hormonal factors contributing to high infanticide in wild male house mice followed by its decrement after mating have not been examined in great detail, but they involve unusually high endogenous testosterone release during neonatal development and a later decrease in circulating testosterone after mating or cohabitation,[82] the latter being similar to numerous other mammalian fathers.

Laboratory Mice

Paternal responding in virgin male laboratory mice is now very often studied, but as discussed earlier in this chapter for the study of female laboratory mice, levels of spontaneous paternal behavior strikingly vary among mouse strains.[83] This indicates the tremendous importance of genetics on males' responses to neonates, but does not make it easy to reach universal conclusions about the endocrine bases of fathering in laboratory mice. Early studies reported that individual differences in circulating testosterone do not correlate well with paternal responses in male Rockland Swiss albino mice,[307] but infanticide is still reduced by castrating males with

no prior killing experience.[308] It has also been seen that neonatally castrated males are *more* likely than males not neonatally castrated to kill pups after being treated with testosterone as adults,[309] suggesting that neonatal testicular hormones render the albino mouse brain to be less responsive to the infanticide-inducing effects of later androgens.

Similar to wild house mice and gerbils (see the section Mongolian Gerbils), mating eventually reduces infanticide and increases paternal behaviors in numerous strains of male laboratory mice that are not already highly paternal as virgins. This effect is maximal soon before their mates give birth and is due to the stimuli associated with ejaculation during copulation. Cohabitation with the mate or another pregnant female is neither necessary nor sufficient for this increase in paternal responsiveness in some strains of mice,[310,311] and in these cases the behavioral change appears to be based only on the number of light–dark cycles experienced by the male after ejaculating.[312] In other strains of mice, both ejaculation and cohabitation with a pregnant female may be needed to promote fathering.[313–315]

The only genetic manipulations of neuroendocrine systems in male laboratory mice that provide information about their paternal behavior involve KO of the progesterone and PRL receptors. The fascinating findings from the PRKO model included a tremendous reduction in infanticide and increased parental behavior by the males, which are of a strain that is normally aggressive toward pups (Figure 51.9).[316] Consistent with these results, wild-type male mice chronically treated with the PR antagonist RU486 are highly paternal,[316] while a history of chronic exposure to exogenous progesterone increases the percentage of males that attack pups even without progesterone "on board" at the time of testing.[317] Progesterone is a primary inhibitor of the onset of maternal behavior in female rats (see the section Hormones Most Significant for the Onset of Maternal Behavior), and this work suggested for the first time that PR activity can also interfere with paternal behavior in a male rodent. With regard to PRL, and in contrast to females, PRL signaling is expendable in male mice and the absence of the PRL receptor gene does not prevent males from interacting normally with pups. These males do not show the PRL-mediated neurogenesis necessary for later recognition of their own adult offspring, but the importance of such late offspring recognition in this species is also not obvious.[318]

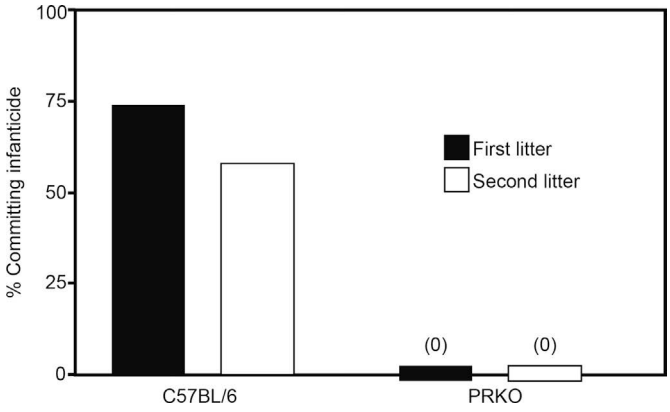


FIGURE 51.9 Percentage of male wild-type (C57BL/6) and progesterone receptor knockout (PRKO) mice committing infanticide after the birth of their first or second litters. PRKO mice do not attack pups. *Source: Modified from Schneider et al., 2003.*[316]

Mongolian Gerbils

Virgin male Mongolian gerbils are either spontaneously paternal[319] or at least become less infanticidal soon after mating.[320] Housing with a pregnant female promotes males' interest in pups,[319] making Mongolian gerbils similar to some laboratory mice and California mice. Like many paternal rodents, circulating testosterone rises in Mongolian gerbils after mating but drops dramatically after pups are born,[321] which is functionally significant because castrating adult virgin males increases paternal behavior, and providing replacement testosterone reduces it.[322] Circulating PRL in male Mongolian gerbils generally rises across their mate's pregnancy and continues to rise as the pups age,[321] although no manipulations of PRL have been conducted to determine any relevance of this change for paternal behavior.

Prairie Voles

Prairie voles (*M. ochrogaster*) are socially monogamous rodents that form lifelong pair bonds after mating and display biparental behavior after the birth of pups. This has been inferred from field studies where both parents are found with offspring in the natal nest or directly observed within seminatural or laboratory environments.[323–326] Plasma testosterone in male prairie voles increases in response to mating and cohabitation with a female,[327,328] and males' paternal behavior then increases across her pregnancy,[328] but it is not known if these events are causally associated.

Unlike unmated California mice, most virgin male prairie voles respond very positively to pups.[329,330] The consequence of castration during adulthood on parenting in these virgin males is equivocal. One study found that half of virgin prairie voles castrated as adults were infanticidal and the remaining voles weakly parental,[331] while a later study reported that castration had no significant effect on parental behavior when the virgin males were tested 4 or 8 weeks after surgery.[330] Differences between the two prairie vole colonies studied in their basal paternal responsiveness (lower in the first study compared to the second) and the presence of a litter effect in the first study probably account for the discrepancy. However, gonadal hormones are not completely irrelevant for

paternal behavior in male prairie voles, but they may be particularly important during early development. Neonatal castration reduces the percentage of virgin male prairie voles acting paternally during adulthood, even when testosterone is replaced weeks before testing.[332] In addition, alloparenting by weanling male prairie voles is reduced by inhibiting aromatization of testosterone to estradiol with 1,4,6-androstatriene-3,17-dione (ATD), or blocking androgen receptors with flutamide, during the second week of life.[333] Consistent with the study showing that adult castration has no effect on paternal behavior in male prairie voles,[330] and very different from studies of male California mice, work by Cushing and colleagues has revealed that neural ERα expression and signaling (presumably from binding estradiol generated by the aromatization of testicular testosterone) has a negative relationship with paternal behavior in prairie voles (detailed in the section Brain Control of Maternal Behaviors). Preliminary data indicate that blockade of progesterone receptors with RU486 has no effect on prairie vole paternal behavior.[2]

The influence of exogenous glucocorticoids on prairie vole paternal behavior is unknown, but Bales et al.[334] found that a single swim stress increases males' later huddling with pups, possibly because males seek out pups as social buffers to dampen their elevated HPA axis activity. Relatedly, stimulation of CRH2 receptors by intraperitoneal injection of urocortin also increases huddling with pups in male prairie voles.[335] A stress-associated increase in huddling with pups may help explain why RU486, which is also a glucocorticoid receptor antagonist, had no effect on pup-directed behavior in unstressed virgin male prairie voles from a colony that was highly paternal.[2]

Only a small amount of research has examined roles of peripheral peptides for paternal behavior in male prairie voles. Preliminary data indicate that their paternal behavior is unaffected by inhibiting pituitary PRL release with the dopamine receptor antagonist bromocryptine.[2] OT is very quickly released into the general circulation when juvenile or adult male prairie voles interact with a pup.[336] Whether this peripheral OT can cross the blood–brain barrier to facilitate ongoing paternal behavior, or is associated with concurrent OT release in the central nervous system, requires further study. Peripheral AVP levels are not affected by pup exposure and corticosterone release is blunted, again indicating that the presence of pups reduces stress.[336]

Dwarf Hamsters

The endocrine basis of caregiving behavior displayed by a subspecies of dwarf hamster that is biparental and monogamous, *P. campbelli*, has been extensively studied. Male *P. campbelli* are transiently alloparental as juveniles but become infanticidal as they age.[337] Reproductively experienced males are unlikely to attack pups, though, and are very paternal by the time their pups are born. In fact, they even contribute to delivery by pulling pups from their mate's vagina and by cleaning the pups of birth membranes and amniotic fluid.[338] Unlike California mice that become more paternal in response to urinary cues from their pregnant mate,[339] the suppression of infanticide and expression of midwifery in *P. campbelli* does not require that males receive any postcopulatory cues from their mates.[340]

Years of detailed work by Wynne-Edwards and colleagues have led to the conclusion that peripheral hormones do fluctuate in *P. campbelli* males across the transition to fatherhood, but that these hormones have very little if any role in regulating their paternal behavior. Correlational work reveals that males' circulating testosterone increases after mating but precipitously drops in concert with a spike in PRL just before pups are born.[341] Furthermore, expression of the long form of the PRL receptor in the choroid plexus falls and then rebounds in anticipation of the pups' birth.[342] Unlike most female mammals, circulating basal estradiol levels in male *P. campbelli* are consistently very high across the peripartum period, and their progesterone levels rise rather than fall associated with birth of a litter.[343] Because testosterone has been thought to impede nurturant behavior,[285] and high PRL and estradiol facilitate mothering in female rats, these patterns of endocrine changes could be reasonable facilitators of fathering in *P. campbelli*. Compellingly, none of these endocrine events occur after mating in the nonpaternal, but closely related, male *Phodopus sungorus*.[344]

While castrating male *P. campbelli* after mating reduces their circulating testosterone and estradiol to almost nondetectable levels, and reduces territorial aggression, it has no effect on retrieval of pups or viability of the litters.[345] Similarly, chronic inhibition of males' estradiol synthesis with the aromatase inhibitor, letrozol, beginning during their mate's pregnancy also has no effect on paternal care.[346] Experimental manipulations of PRL have revealed similar negative results—dopamine D2 receptor antagonism that reduces circulating PRL a few days before and after the birth of pups has no consequence on paternal behavior in *P. campbelli* (Figure 51.10).[347]

In sum, there appears to be a pattern of low testosterone and high PRL in *P. campbelli* and many other rodent fathers, and these hormones can sometimes be correlated with particular aspects of their paternal behavior. These correlative relationships are not found in all rodent fathers, and the cause-and-effect relationships between circulating hormones and paternal behavior in some species remain to be examined.[348,349] In species where experiments have been conducted to manipulate

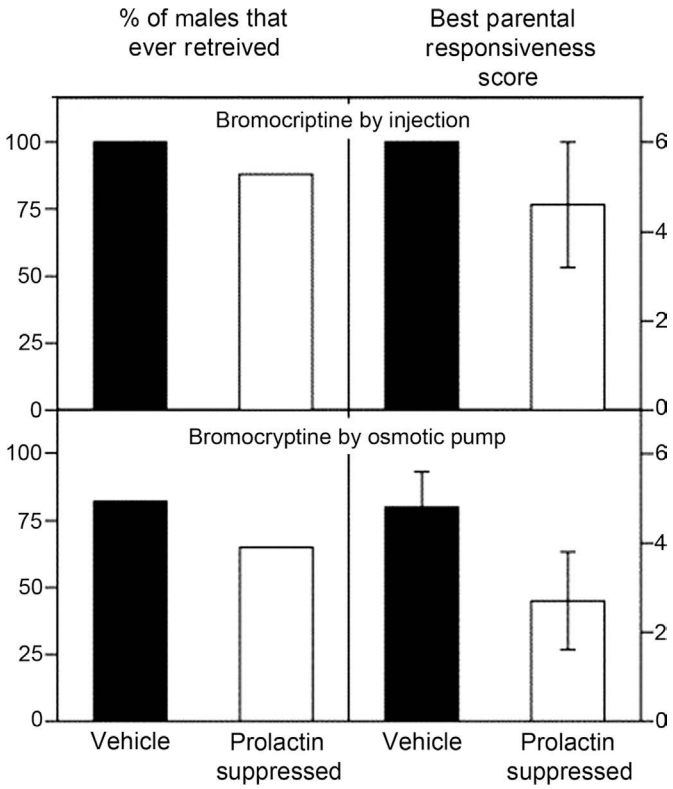


FIGURE 51.10 Percentage of male Djungarian hamsters that ever retrieved a pup, and their highest overall parental responsiveness score, after administration of vehicle or bromocryptine for the 3 days before birth of their pups via subcutaneous injection (top panels) or chronically via osmotic minipumps (bottom panels). Prolactin suppression had no effect on paternal responding. *Source: Modified from Brooks and Wynne-Edwards, 2005.*[347]

males' endocrine status, paternal behavior may or may not be affected. Thus, there is no universal endocrine basis of fathering in paternal rodents, suggesting that the behavior has evolved numerous times via numerous endocrine and nonendocrine mechanisms.

Primates

The small arboreal New World monkeys, the Callitrichids, are the best studied nonhuman primates for examining relationships between hormones and paternal behavior. They are cooperative breeders that live in family groups consisting of a monogamous male–female pair and their offspring of various ages, all of whom help care for the youngest infants in the family.[350] Paternal involvement in some Callitrichids and other nonhuman primates is so impressive that fathers may be the primary nonnutritive caregiver and can be observed to carry their infants considerably more than the mothers![351,352]

Testosterone and PRL were the first hormones examined for an association with paternal care in any primate. Dixson and George reported that most, but not all, male common marmosets (*Callithrix jacchus*) cohabitating with their mate and young offspring had lower circulating testosterone compared to males living with a nonpregnant or a pregnant female.[353] This result was bolstered by later work showing lower testosterone in common marmoset fathers after the birth of the infants compared to beforehand and a drop in males' testosterone during acute exposure to infant-related cues.[354,355] Testosterone was also inversely associated with infant carrying in black tufted-ear marmoset fathers (*Callithrix kuhlii*).[356] On the other hand, there is no significant relationship between circulating testosterone and paternal behavior in white-faced marmosets (*Callithrix geoffroyi*),[357] although this does not eliminate the possibility that the relevant testosterone is converted to estradiol within the brain to influence their behavior.[292]

Along with their decreased testosterone, common marmoset fathers sometimes have tremendously high circulating PRL.[353,354] PRL levels are elevated in these fathers only if they very recently carried an infant, and the levels are positively correlated with how long they carried the infant; these findings are not specific to fathers because they occur in male marmoset alloparental "helpers."[353,358–360] In contrast to marmosets, father Goeldi's monkeys (*Callimico goeldii*) do not have higher PRL levels than their adult nonreproductive sons, but PRL in fathers rises after the birth of their neonates and drops after they begin expressing paternal behavior.[361] Also different from marmosets are Pithecid fathers (Titi monkeys; *Callicebus cupreus*), which have higher urinary PRL compared to their nonreproductive sons a few weeks before the infants' birth, but show no changes in urinary PRL from a few weeks prepartum to 3 weeks postpartum, even though they carry their infants almost exclusively.[361]

In rare experimental studies manipulating hormones in monkey fathers, it was found that suppressing pituitary PRL release reduced infant carrying by alloparental marmoset males,[362] but that experienced fathers showed no such reduction in carrying after PRL suppression and were instead slightly more interested in infants compared to controls.[363] In fact, experience is an important determinant of whether endocrine fluctuations even occur in some nonhuman primate fathers. For example, there is no prepartum to postpartum hormonal change in mated male cotton-top tamarins (*Saguinus oedipus*) that have no parenting experience, but experienced males undergo rises in estrogens (estradiol and estrone), androgens (testosterone and dihydrotestosterone), PRL, and cortisol as their mate's pregnancy progresses, perhaps triggered by olfactory or other cues from the female.[364] These hormonal changes in male fathers can have important peripheral effects, in addition to any central effects. This can be seen in male tamarins that experience a PRL-mediated increase in body weight across their mate's pregnancy, which may be necessary for the metabolic demands of later infant carrying.[354]

The only nonhuman apes that exhibit paternal care are members of the family Hylobatidae, which consists of the socially monogamous and pair-bonding "lesser apes," commonly known as gibbons and siamangs. They provide an interesting comparison between closely related species because gibbons mostly ignore infants while siamangs readily carry their offspring.[365] In a study involving small numbers of three species of paternally experienced Hylobatidae, fathers' fecal androgen and estrogen metabolites did not significantly change from 1 month prepartum to 7 months postpartum in the nonpaternal gibbons, but androgen metabolite levels in the paternal siamangs rose across the mate's pregnancy and decreased after she gave birth. Males' estrogen metabolites also increased across pregnancy and were maintained at high levels through the postpartum period,[365] which is reminiscent of changes in estrogenic activity in paternal California mice.

It is probably unnecessary to state that many human fathers can be engaged and engrossed with their infants to the degree typically found in mothers.[366] Intense paternal behavior is common in humans, but not ubiquitous, with fathers having moderate to high contact with young infants in about half of the cultures examined.[367] As with naturally paternal rodents, high paternal involvement in humans is most likely to be found in cultures characterized by monogamy in which assurance of paternity is relatively high. It is also more likely to be found in cultures that conduct only small-game hunting and where animal husbandry is absent—the cultures perhaps with the least reliable food resources in which paternal assistance can have the greatest impact on offspring survival.[367] Even so, paternal involvement in childrearing other than basic "breadwinning" is still highly variable within most cultures where fathering commonly exists.[279] Furthermore, in societies where paternal interaction with infants is considered high, the average duration of time that fathers spend each day in physical contact with their infant or toddler can be quite small (15–90 min per day in the United States), even though fathers' general availability to offspring is usually considerably higher.[368] It is also true that human paternal behavior differs from maternal behavior in numerous ways, including that fathers are more arrhythmic in their interactions with infants, are more physically stimulating to them, respond more to infant motor cues than social cues, and are most likely to be touching their infants when play is occurring than during other types of interaction.[366]

Research on the endocrinology of human fathering has often hypothesized homology across species and between the sexes, and so predicts that fathers will have low circulating testosterone but high estradiol and PRL. In the first study of its kind in a now-burgeoning literature, Storey et al.[369] used a longitudinal design to find that plasma testosterone levels in men did drop after their partners gave birth, and others soon thereafter

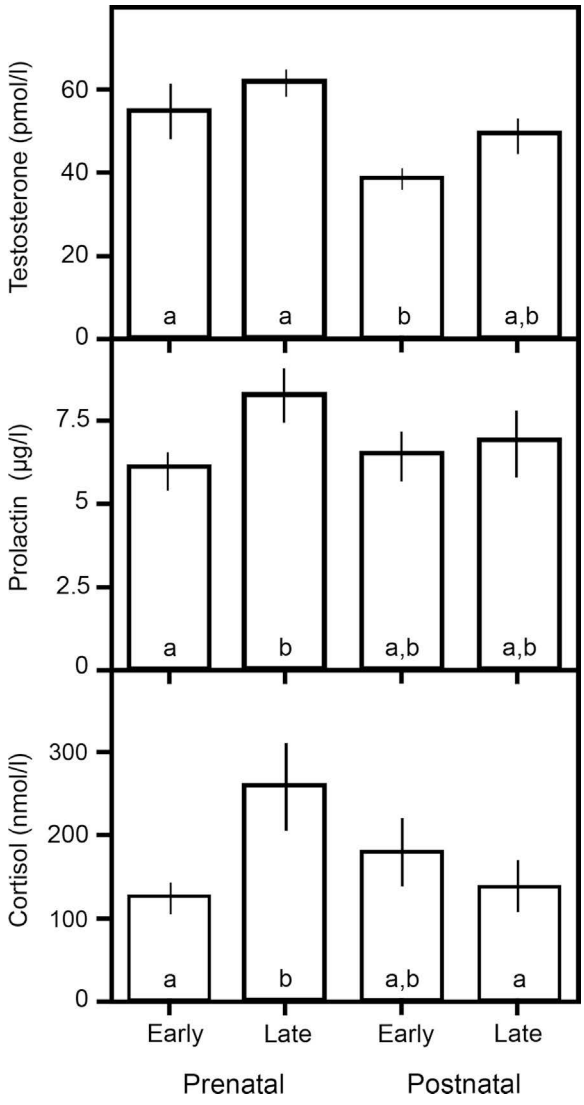


FIGURE 51.11 Plasma concentrations of testosterone, prolactin, and cortisol in human fathers before and after the birth of their infant. Different letters at base of bars indicate significant differences compared to the early prenatal group. *Source: Modified from Storey et al., 2000.*[369]

reported similar results (Figure 51.11).[370] Later studies in numerous cultures indicate significantly or at least notably lower testosterone in partnered fathers compared to partnered nonfathers or unpartnered nonfathers[370–375] and this difference is greatest when they are compared against the most involved fathers.[372] Even though human fathers' baseline testosterone is lower than that of nonfathers, fathers respond acutely with a rise in testosterone after hearing infant cries, although this acute increase is less in men expressing the greatest sympathy and concern to those cries.[369,371] In contrast, testosterone levels fall in fathers during a 30 min face-to-face interaction with their 2 year olds, but this depends on the context because it is only found in the fathers who spend *more* time interacting with the child during testing and

whose partners spend *less* time interacting with their toddler during the test, and only if it is a day when fathers have already spent considerable time with their children.[376] An important consideration for evaluating the results of these studies is that testosterone concentrations are stably lower in men who eventually pair bond than in single men who do not form such a relationship,[377] so the longitudinal studies and the studies comparing partnered fathers with partnered childless men are particularly valuable for determining relationships between men's testosterone and their fathering status or behavior.

Estradiol also changes across fatherhood in men. A longitudinal study of men becoming fathers for the first time found that estradiol was more likely to be detected in their saliva samples than it was in samples from nonfathers. Fathers' estradiol was also more likely detectable a month after the infant was born compared to a few weeks before the birth, even though absolute levels of estradiol did not significantly differ in men before and after their infant's birth.[370]

PRL levels in men rise across their partner's pregnancy,[376] resulting in fathers having higher PRL than nonfathers, particularly for men who are first-time fathers or are fathers of very young children.[371,378] Furthermore, PRL levels are correlated with paternal responsiveness, with greater PRL levels in men who report more alertness to and concern with recorded infant cries, as well as who show greater attention and coordination in their behavior with the infant.[369,371,379,380] Levels of PRL drop while first-time fathers interact with their child, though, indicating that not only do the most paternally sensitive men have the highest PRL but their hormones probably predict their upcoming behavior rather than are a result of it.[371,376,379,381] An interesting parallel is found in male meerkats, where PRL levels predict which males are the most likely to initiate a bout of caregiving.[382] Fascinatingly, PRL is also higher in fathers who report more couvade symptoms, the sympathetic somatic and emotional events characteristic of females' pregnancy, further reflecting these men's greater investment in the upcoming birth and higher sensitivity to their partner's experience.[369]

There is no evidence that circulating or plasma OT is higher in fathers than nonfathers, which is also true for postpartum women who have not recently breastfed, but OT levels in fathers are associated with emotional synchrony, engagement, and positive communication with the infant.[200,380] These men with high OT are prone to this type of social interaction because they also report greater attachment in social relationships throughout their lives, including with their parents and romantic partners.[200]

Perhaps related to apprehension about the upcoming birth of an infant, glucocorticoid levels rise in expectant fathers during their partner's late pregnancy and peak the week before the delivery.[369,370] Similar to PRL, these high glucocorticoid levels drop across time while fathers interact with their infants[381] or toddlers.[376] Fathers with showing the smallest drop while interacting with their toddler are the most attentive fathers,[376] which is similar to the greater maternal responsiveness sometimes found in postpartum women with higher cortisol,[232] again suggesting that elevated cortisol at reasonable levels can positively contribute to parental attention. Because glucocorticoids (and PRL) have a reactive component to their release, these studies can be difficult to interpret and depend on the environment in which the samples were taken and the possibility that interacting with an infant can itself be acutely stressful.

In sum, unlike studies of rodents, the extant studies of primate fathers are generally consistent among each other, and the observed hormone profiles in these fathers generally support the hypothesis of homology between the sexes in their endocrine release before and during parenthood. This is particularly true for PRL. Given that, there are conflicting data regarding whether there are significant correlations between the hormone levels of individual father-and-mother pairs.[200,369,383] As with our discussion about the relevance of the correlations between hormones and behavior in human mothers (see the section Hormones Most Significant for the Onset of Maternal Behavior), a greater understanding of how and how much these changes in circulating hormones affect paternal behaviors in primate fathers is a critical area of future research.

SENSORY CONTROL OF MATERNAL CARE

Parents must accurately recognize infants at either short or long distances to initiate contact seeking and establish the physical proximity required for the execution of parental duties. As already noted in the Introduction, parenting is not a unitary process but is composed of numerous individual behaviors, and these behaviors differ in what sensory inputs elicit and maintain them. Studies of the sensory control of parenting traditionally focus on the senses involved in maternal recognition of young and contact-seeking behaviors, rather than the senses required to maintain or terminate physical proximity after its establishment. In laboratory rodents, the senses involved in maternal approach to offspring, followed by carrying them to the nest, are most often studied. Although retrieval is probably rare in undisturbed environments with young, mostly immobile pups, how rapidly dams approach offspring during a retrieval test can provide particularly valuable information about factors modulating maternal motivation to initiate contact with pups, as well as the ability to pick them up, hold them in the mouth while transporting them, and finally release them in the nest.

In some of the earliest studies investigating the sensory requirements for retrieval in multiparous postpartum laboratory rats, Beach and Jaynes[32] reinforced the important concept of "multisensory control" for how the senses act individually and together for a mother's behavioral responding. Dams appeared to use both olfactory and visual cues to orient toward pups, but retrieval remained rapid after cauterizing the olfactory bulbs and was only slightly slowed by surgical removal of the eyes. Furthermore, loss of tactile input (i.e., anaptia) by severing any one of six sensory nerves subserving the face and head only slightly affected dams' retrieval capabilities. A small number of animals tested after two desensitizations displayed somewhat greater impairment, and only after three desensitizations (blind–anosmic–anaptic) did performance notably suffer, even though some still retrieved a few pups. From these results, it was concluded that dams may normally use all available senses to fine-tune their behavior, but any individual sensory modality was mostly expendable as long as there was sufficient input from the other senses to compensate. More recent studies on this topic from primiparous laboratory rat and human mothers discussed below support many of Beach and Jaynes's early conclusions.

Vision and Hearing

Vision and hearing are still thought to be mostly unnecessary for maternal behavior in rats. Rats that are enucleated before giving birth or had their eyes temporarily sutured closed after parturition are competent mothers and raise healthy pups.[384,385] Consistent with this, blind nulliparae are just as likely to become maternally sensitized as sighted ones,[386] all of which may not be surprising for this nocturnal, burrowing rodent. With regard to sound, mother rats and mice can detect pup sonic and ultrasonic vocalizations, and can discriminate the sex of the pup based on their vocalizations,[387] but will not use sound alone to guide their behavior.[388,389] Dams also do not absolutely require sonic inputs, and deaf postpartum or Caesarean-sectioned rats still retrieve pups although do so more slowly compared to hearing females.[15,384] Similarly, dams orient to and retrieve dead, chilled, or anesthetized pups but do so more slowly than when presented with active and vocalizing pups.[390–392] Deaf postpartum rats also exhibit normal licking and nursing,[385] and deaf virgins are just as likely to become maternally sensitized as hearing females, but are more likely to accidentally step on and injure a pup.[386] The combination of blindness and deafness decreases nursing frequency in rats, but is not inconsistent with rearing viable pups.[393]

Diurnal ungulates living in large, mobile groups can also dispense with visual or auditory cues for maternal identification of or behavior toward their lambs. Blindfolded ewes and beef cows can identify and accept their young,[394,395] and ewes continue to prefer the vocalizations of their own lamb even if they cannot see them.[396,397] This does not mean that auditory cues from the lamb are what is required for maternal identification, because ewes successfully find their lambs even after surgical removal of the ewe's auditory canals[394] or when the lambs are contained in a soundproof chamber.[398] In both cases, the ewes may act by sight, although ewes are unable to readily learn to use photographs of their lamb's faces as discriminative stimuli to gain access to their offspring.[399] Importantly, when both vision and hearing are eliminated, ewes are much less likely to immediately choose their own lamb over alien lambs. Moreover, most ewes cannot find any lambs at all when the sense of smell is simultaneously impaired with vision and hearing.[394] It was once thought that at short distances from the lamb, sound and sight have little role in ewe's acceptance of the lamb at the udder and that olfaction was primarily responsible,[394] but this has more recently been questioned after the finding that anosmic ewes do recognize their own lambs within hours after giving birth and later use the remaining available cues to selectively nurse them.[400]

How a lack of vision influences maternal care in human and nonhuman primates has rarely been studied. This is surprising because human mothers and infants are innately interested in each other's faces, normally spend a tremendous amount of time looking at each other, use eye-to-eye contact and its disengagement to modify their interactions, and the foundation of their bonding usually involves face-to-face interactions.[401,402] After spending as little as an average of 5 h with the neonate, mothers (as well as fathers) can accurately identify photographs of their infant's face.[403,404] These infant visual cues, including the infant's physical attractiveness, are unconsciously associated with differential maternal responses to and attributes made about the infant.[405] In the absence of vision, however, maternal interactions with infants appear to be normal, and blind mothers use a variety of flexible strategies to interact and communicate with their infant.[406,407] Compared to the study of maternal vision, maternal hearing has received more investigation. Most human mothers distinguish between the cries of their own and other infants within 48–72 h after parturition, and many can do so even before then.[408,409] Some analyses of deaf mothers interacting with their hearing infants indicate no differences,[410] while others report that deaf mothers have less vocal interaction with their infants,[411] and yet others find that deaf mothers vocalize more often than do hearing mothers during face-to-face interaction with their hearing infants.[412] In the absence of incoming auditory cues, deaf mothers may compensate by briefly touching the infant and using hand gestures more often, which is thought to help maintain the connection with and attention by the infant.[413,414]

Olfaction

Over the past four decades, there has been a tremendous amount of research devoted to understanding the olfactory control of maternal behavior in rodents, consistent with the thought that olfaction is the primary sensory mediator of social information in nonprimate mammals.[415] A distillation of this literature is that olfactory cues from rat pups are unattractive or even aversive to nonpregnant or nonparturient rats, and that a primary consequence of peripartum ovarian and pituitary hormones is to alleviate this olfactory aversion and permit attraction to the sensory cues from neonates. After hormones have completed this task, olfaction is thereafter mostly unnecessary for ongoing maternal care. These hypotheses were partly based on anecdotal observations of differences in behavior of parturient and nonparturient females after young pups are placed in their home cage.[10,416] While mothers rapidly seek out and retrieve or lick the pups, nonparturient females exposed to pups for the first time approach and sniff them, but usually make a hasty retreat. Nulliparous females also actively avoid previously preferred parts of their home cage if pups are placed there,[417] but such avoidant responses wane after a few days of exposure, and maternal responses often emerge thereafter.

Early work from Fleming and colleagues provided further supporting evidence that olfaction inhibited mothering in rats. They found that virgin female rats exhibit a rapid onset of maternal behavior after being olfactory bulbectomized, with latencies of ~2–3 days versus ~7 days in controls.[418] Moreover, 70% of bulbectomized responders were maternal within 24 h after their first exposure to pups. In subsequent studies, they further demonstrated that peripheral anosmia induced by irrigating the nasal cavity with zinc sulfate also elicited rapid mothering in most virgin females and that sectioning the vomeronasal nerve reduced sensitization latencies by 50%.[419] Because combining vomeronasal section with olfactory bulb damage stimulated the behavior even faster (Figure 51.12),[420] both pheromonal and volatile olfactory appeared to inhibit maternal responding. Similar hastening of caregiving behaviors in virgin rats occurs after destroying the medial amygdala (MeA), stria terminalis, or bed nucleus of the accessory olfactory tract,[421,422] which transmits olfactory information to the hypothalamus and other areas of the basal forebrain. Disruption of olfactory paths even promotes maternal responding in virgin male rats,[423,424] which are less positively disposed to pups than are virgin females.[2] Although maternal responding in olfaction-disrupted nulliparous rats is impressively hastened, it is not the only inhibitor of mothering because administration of ovarian hormones makes anosmic females even more responsive to pups.[425]

A number of points are relevant for interpreting the literature on olfaction and mothering. First, the ability

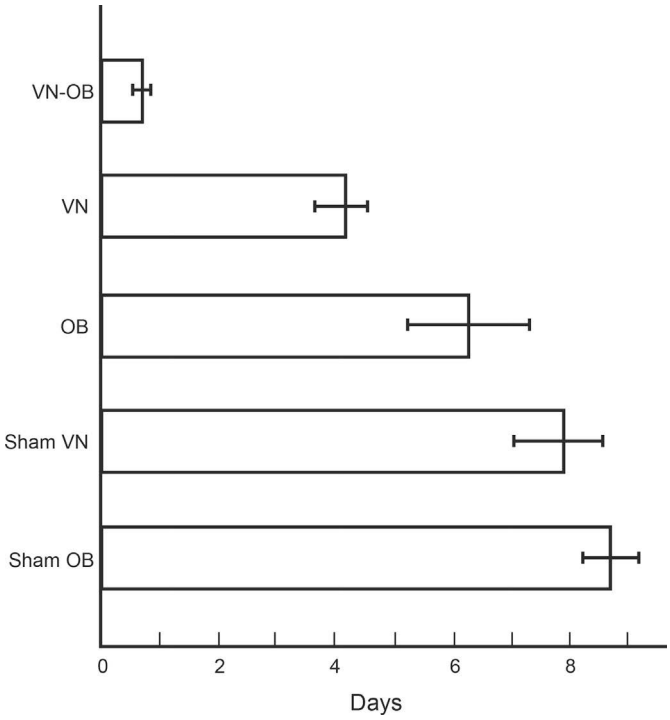


FIGURE 51.12 Mean latency for nulliparous female rats to display maternal behavior after receiving cuts to the vomeronasal nerve (VN), olfactory bulb (OB), or both (VN–OB). Control animals received a sham section of the VN or OB. Note the particularly fast sensitization in the VN–OB females. *Source: Modified from Fleming et al., 1979.*[420]

of intranasal irrigation of zinc sulfate to produce anything other than a brief (<72 h) and incomplete anosmia in rats has long been questioned.[426,427] In fact, one early study indicated that the loss of olfactory discrimination after intranasal zinc sulfate did not correspond well with maternal responding, given that most females were probably no longer anosmic 2 days after treatment but still exhibited very rapid onset of maternal behavior at that time.[428] Second, the concept of segregated main and, accessory olfactory systems has been questioned because they functionally overlap more than is often considered. For example, both systems can respond to volatile odors and pheromones, and the difference between them is in their degree of responsiveness to particular odorants, rather than their ability to respond to them at all.[429,430] Third, olfactory disruption in mammals broadly affects endocrine function, including thyroid, pineal, pituitary, gonadal, and adrenal hormones.[431] Olfactory bulbectomy also increases the expression of estradiol receptors in the female rat MeA,[432] which may contribute to faster sensitization; there is a relationship between ERα expression in numerous other brain areas and the propensity to sensitize in olfactory-intact virgin female rats.[433] Whether or not such changes in hormone release or expression of their receptors occur in a time frame consistent with studies of maternal behavior that can initiate pup exposure days or weeks after a manipulation

should be considered. Interestingly, Gonzales-Mariscal and colleagues found that disrupting main or accessory olfactory input dramatically hastens maternal responsiveness in virgin rabbits, but not if females were ovariectomized.[434,435] A similar need for the ovaries is found for the facilitated maternal behavior after cell-body-specific chemical lesions of the MeA in nulliparous rats.[436] Lastly, if olfaction is a primary inhibitor of maternal responding, it is surprising that sensitization is not necessarily hastened in nulliparous rats by habituating them to distal cues from pups encased in a wire mesh box and placed in the home cage before sensitization begins.[437,438] On the other hand, female rats reared from birth to weaning in a colony room that housed other dams with pups do sensitize faster during adulthood than females raised without this exposure.[439] This could indicate that exposure to distal pup cues, especially during early development, establishes a strong, long-lasting habituation to these cues compared to what can be achieved when exposure occurs during adulthood.

It is also valuable to note that two studies sometimes cited as evidence that mothers are attracted to pup odors while nonmothers find them aversive did not demonstrate report data leading to that conclusion. Bauer reported that late-pregnant and early-postpartum rats preferred their own soiled bedding, or bedding soiled by other postpartum rats, over bedding soiled by virgins or clean bedding.[440] Because peripartum females also equally preferred bedding of late-pregnant rats, the source of the preferred odor could very well be from mothers rather than from pups. Furthermore, nulliparous virgin females in this study did not have an aversion to soiled bedding from maternal nests, but rather had no preference for it over clean bedding. Similarly, Fleming et al. reported that steroid hormone–primed nulliparous rats had a strong preference for soiled nest material over clean bedding, but the control females given cholesterol did not show an aversion to the soiled bedding—if anything, they had a slight preference for it.[441]

Any need for olfaction during ongoing postpartum maternal behavior in rats is unclear. Most studies find little effect of olfactory bulbectomy performed before mating or during pregnancy, but a few have reported high pup mortality at parturition or impaired maternal behaviors thereafter (see Refs 442–444). Discrepancies might be explained by the nonsensory consequences of bulbectomy because neither peripheral anosmia nor vomeronasal nerve damage has much effect on postpartum pup care.[442]

In contrast to its inhibition of mothering in virgin rats and likely irrelevance in postpartum rats, olfaction is essential for maternal behavior in female mice. Because most strains of virgin laboratory female mice are spontaneously parental to varying degrees, it must be the case that no pup sensory cues invariably need to be overcome. Both virgin and postpartum mice can use pup odor to find and retrieve them, and olfactory bulbectomized virgin mice show no maternal care and often cannibalize pups.[389,445,446] Bulbectomy before mating, during pregnancy, or postpartum produces similar effects in parturient females.[447–449] Less dramatic impairments in mothering are observed after peripheral anosmia with zinc sulfate,[449] and maternal experience can mostly compensate for this peripherally induced loss of olfaction[450] but it cannot overcome bulbectomy.[447] Inducing general anosmia by deleting the *SCN9A* gene in olfactory sensory neurons, thereby eliminating the Nav1.7 voltage-gated sodium channel, abolishes retrieval but apparently does not induce cannibalism.[451] This genetic manipulation helps clarify the effects of anosmia on mouse maternal care by avoiding the known side effects of surgical intervention or nasal irrigation with zinc sulfate. The importance of the main olfactory system in mouse mothering seems to also be supported by the severely impaired retrieval and nest building of virgin or postpartum mice with a targeted deletion of the adenylyl cyclase type 3 (*AC3*) gene,[452] which is necessary for second-messenger cascades in main olfactory neurons, but presumably also in any of the brain sites underlying maternal behaviors.

Vomeronasal organ removal in female mice leaves maternal behavior intact,[453] as does eliminating a suite of putative pheromone receptor genes.[454] However, deleting the *Trpc2* gene that codes for an ion channel found in the vomeronasal organ deforms the accessory olfactory bulb, reduces the frequency of postpartum nest building and nursing,[455] and results in an early decline in nest attendance as postpartum time ensues.[456]

Olfactory control of mothering in ewes involves a prepartum inhibition perhaps similar to rats, followed by a postpartum facilitation that differs in purpose from that found in mice. Amniotic fluid is aversive to nulliparous female sheep, but the aversion can be relieved by peripheral anosmia with zinc sulfate or, of course, by the hormonal milieu of pregnancy.[457] Mothers' strong attraction to amniotic fluid at parturition is observed even toward a bowl containing the liquid.[458] Ewes reject and are even aggressive toward their own lambs if the offspring are washed immediately after delivery,[459] and they are more prone to accept alien lambs wearing a jacket soaked in amniotic fluid.[460] When examined immediately after parturition, the lack of olfaction produces small detriments to, but does not prevent, mothering in primiparous ewes and has no effect in multiparous ewes.[461] Anosmic ewes do show indiscriminate mothering toward any lamb and[461] anosmic postpartum goats are similarly indiscriminate.[462] Severing the vomeronasal nerve does not produce such effects in ewes, so main olfactory cues are probably more relevant than pheromones for early maternal responding and identification of the lamb.[461]

The enlarged cortex of primates has diminished the importance of olfactory communication to guide their behavior,[229] resulting in only a small literature on olfaction and primate mothering. This is despite the fact that body odors can both convey and influence emotional states in humans and other primates,[463] which helps set the stage for maternal caregiving (see the section Sensory Control of Maternal Care). It is well known that infant human and nonhuman primates are attracted to and can identify their mother by her olfactory cues alone, and use those cues to guide their contact-seeking behaviors.[464,465] In the reverse direction, early postpartum human mothers find the odor of T-shirts worn by infants more attractive than nonmothers do, and this attraction is associated with individual differences in maternal behavior—mothers who rate their infant's odor as highly attractive spend more time in close physical contact with their infant than do mothers who give lower ratings.[466] Women can also use the sense of smell to selectively identify clothing worn by their infants even after surprisingly little interaction with them after parturition,[467,468] but maternal recognition of her own odor profile, which may be inherited by infants, could contribute to this ability. It would be fascinating to study if the inability to detect infant odors disrupts human mothering, but anosmia in reproductive-aged women is uncommon conditions that do not otherwise compromise reproduction and parenting (e.g., Kallman syndrome, schizophrenia, and multiple sclerosis). There are no readily found studies of mothering by anosmic monkeys, and one might predict that the absence of olfaction would have little consequence.[469]

Taste

It has not been examined in detail if taste influences any maternal behavior in any species, but anesthetizing the tongue significantly decreases maternal licking but does not affect retrieval.[470] This finding is difficult to interpret because tongue anesthesia impairs both taste and somatosensory inputs, and may impede motor control of the tongue by the hypoglossal nerve in a way known to reduce other types of licking (i.e., nonpup).[471]

Somatosensation

After being drawn to their offspring via distal cues, perioral and ventral somatosensory inputs that mothers receive from the pups are essential for the normal pattern of mothering to continue (Figure 51.13).[73] Retrieval is inhibited if the texture of pups' skin is altered by making it greasy or tough,[32] or if the pups' skin temperature is abnormally low,[390] but not if it is too high.[472] Dams detect these pressure and thermal skin qualities through the infraorbital branch of the trigeminal nerve—which mediates the tactile function of the snout, whiskers, nose, and mucous membranes of the mouth—in order for the mouth-opening reflex that is needed for retrieval to occur. Reducing or eliminating tactile inputs to the perioral region by anesthetizing dams' mystacial pads or severing the infraorbital nerve impairs or even abolishes retrieval and licking of pups.[73,473] Such deficits are not observed after severing the mental nerve serving the chin and lower lip. Importantly, although dams with infraorbital denervation or anesthesia cannot retrieve, they still self-groom (and even do so more than controls), indicating differential sensory control of retrieving and some other oral behaviors.[73]

The mostly abolished retrieval and licking seen after perioral anesthesia or nerve cuts are greatly lessened if rats have pretreatment experience retrieving pups or trying to retrieve pups under previous lidocaine treatment,[473,474] suggesting compensation by other sensory inputs. Indeed, experienced dams spend additional time sniffing pups between retrievals, perhaps to maximize the input from remaining sources of stimulation.[474] The maternal experience of multiparous females could explain why early studies indicated little effect of sensory denervation of the snout or mouth on rat mothering.[32] This is reminiscent of the effects of olfactory bulbectomy on postpartum behavior, which, when found, are diminished in multiparous compared to primiparous dams.[475]

Perhaps unexpectedly, Stern and colleagues found that perioral tactile inputs facilitate not only dams' oral maternal behaviors, but also their quiescent nursing.[470] Dams separated from their litters for 4–6 h and then subjected to perioral anesthesia shortly before reunion did not continually attempt to interact with pups but ignored the individual offspring scattered around the cage. Even if the litter was manually placed directly in the nest, perorally anesthetized animals still ignored the pups and rarely nursed them. Thus, just having the pups in a single location without tactile input to the dams' snout is insufficient to maintain maternal interest or eventually lead to nursing of young pups. Because older and more motorically capable pups are persistent in seeking out their mother and can burrow under their perorally desensitized dam to search for a nipple, nursing can be elicited. Interestingly, if anaptic dams are deprived of contact with offspring for a rather long period of time before testing (~24–36 h), mothers do remain with a young foster litter and nurse,[476,477] suggesting that high maternal motivation overrides the lack of perioral inputs for nursing to occur.

The skin of the snout is not the only tactile regulator of mothering in laboratory rats. Nursing behavior, involving cessation of motor activity and often kyphosis (crouched posture over pups), is closely tied to the

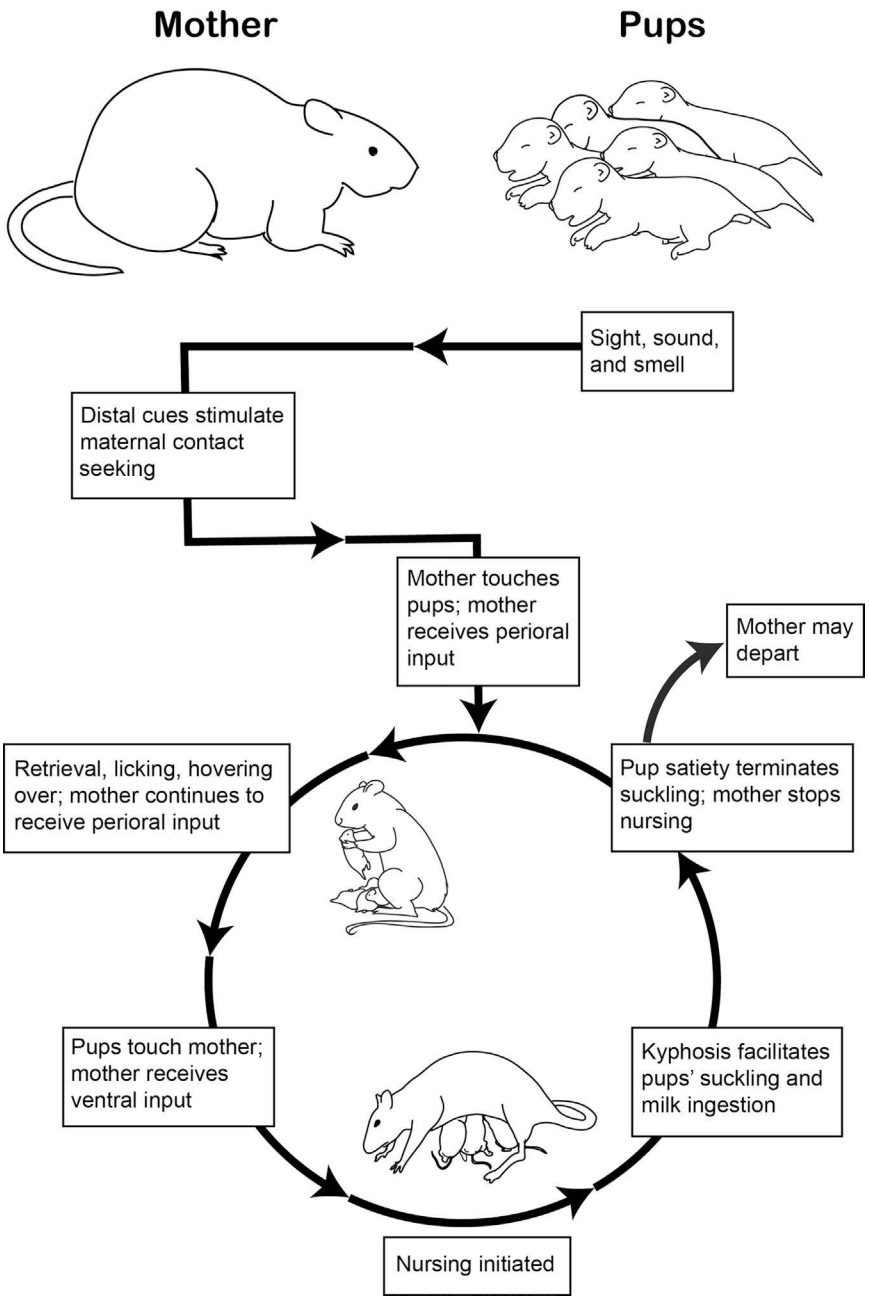


FIGURE 51.13 Sensory events regulating maternal behavior in laboratory rats. In this dyadic interaction, distal cues from pups elicit maternal contact seeking, which then permits the mother to receive perioral tactile inputs required for retrieval. Perioral tactile cues that dams receive while in the nest, mostly obtained while licking the pups, maintain her interest in interacting with the litter and stimulate the litter to search for nipples. Once a sufficient number of pups attach to nipples and begin suckling, these ventral inputs that the dam receives elicit her to show prolonged bouts of quiescent nursing and eventually let down milk. Sated pups will detach from nipples, and the loss of suckling inputs will often result in either the dam resuming active maternal behaviors while hovering over the litter or the dam leaving the nest. *Source: Modified from Stern, 1996.*[73]

somatosensory inputs that dams receive to their ventrums while pups search for and suckle on a nipple. This requisite ventral stimulation must come from a relatively large number of active pups. Indeed, dams are unlikely to nurse if interacting with fewer than four pups or if the pups are immobilized by anesthesia, chilling, or warming.[73] Furthermore, the pups must be not only active but also capable of suckling because normal nursing cannot occur if the pups' mouths are closed shut or anesthetized, or if the dams cannot detect pup suckling because her ventrum is anesthetized or her nipples were surgically removed (a procedure termed *thelectomy*).[390,478] In the absence of adequate ventral stimulation and the quiescence it instills, mothers often continue to actively hover over the litter while exhibiting oral behaviors (licking, repositioning the pups within the nest, and self-grooming) until eventually lying flat on top of the litter without assuming kyphosis or simply leaving the nest.

The termination of a nursing bout also depends on sensory inputs from the pups. The intensity and rhythm of pup suckling reflect their nutritional state, with hungry pups attaching to nipples faster, maintaining attachment longer, and sucking more vigorously and synchronously than do sated pups.[479,480] Because pup suckling induces dams' quiescence, more suckling

results in longer nursing bouts. If hungry pups are suckling a dam that cannot provide milk because her nipples are ligated, or because she is treated with the PRL release inhibitor bromocryptine, nursing bouts are prolonged.[73,481] Conversely, if pups are artificially fed through a gastric tube before interacting with their mother, nursing is truncated.[386] Termination of nursing behavior in rabbits, which typically occurs once per day for only a few short minutes, is also at least in part controlled by suckling inputs from the young.[59]

Given the importance of suckling for the initiation and continuation of nursing bouts in lactating rats, it is surprising that some aspects of the nursing-like behaviors displayed by sensitized nulliparous rats (which are naïve to pregnancy's endocrine influences on skin sensitivity and have no developed nipples upon which pups can suckle) are more similar to those of suckled postpartum dams than those of postpartum thelectomized rats.[107] Perhaps once a postpartum female rat experiences suckling pups, the range of sensory inputs required for quiescence and nursing behaviors is narrowed, but maternal virgins without such experience remain responsive to nonsuckling inputs from the litter.

A number of studies have revealed a critical role for somatosensation in establishing and maintaining maternal responsiveness more generally. One of the earliest events indicating upcoming successful maternal sensitization in nulliparous rats is not a sudden onset of retrieval, but females' tolerance of physical contact with pups that had crawled to them.[386,482] This is consistent with the unusually fast sensitization of virgin rats housed in small cages that do not allow her to avoid physical contact initiated by the pups.[482] In mated female rats, if physical interaction with pups at parturition or relatively soon after a Caesarian delivery is prevented, the mothers are later unresponsive to pups even if they had earlier received distal cues from them.[437,483,484] This early somatosensory input that establishes later interest in pups is diffuse and can be satisfied by either perioral or ventral inputs. If both sources are blocked during the initial interaction with pups, via perioral anesthesia combined with a "jacket" covering the trunk, females are as unresponsive to pups as Caesarian-sectioned females who have never before seen pups at all.[477]

Tactile sensitivity of the human breast, nipples, and areolae is higher during the first few days postpartum compared to during pregnancy.[485] It is unknown if this is also true for other highly sensitive skin, such as the fingertips or face, but it is not found on the back of the hand.[486] The power of this sensitivity is indicated by the ability of postpartum women to identify their own infant just by stroking the infant's hand.[487] Such changes in tactile sensitivity are surely the consequence of ovarian and other hormones,[488,489] and are important for successful breastfeeding and perhaps early bonding between mothers and infants.[490,491] Disorders associated with tactile hypersensitivity (e.g., fibromyalgia or chronic pain) or hyposensitivity (e.g., multiple sclerosis or spinal cord injury) may be expected to interfere with mother–infant interactions through both sensory and cognitive mechanisms, but this remains to be extensively studied.

These findings about how the tactile senses are critical regulators of mothering in laboratory rats are not necessarily universal. For example, the inability to touch the lamb after parturition has little effect on ewes' later maternal acceptance of them as long as they had been able to smell the lambs at a distance.[492] Another possible example is related to the fact that rabbits do not retrieve displaced young and rarely lick them during their brief once-daily interaction,[493] so presumably the absence of perioral sensations would have little or no effect on rabbit mothering.

BRAIN CONTROL OF MATERNAL BEHAVIORS

Brain Control of Maternal Behaviors in Nonhuman Animals

Most of what we know about the neurobiological mechanisms regulating maternal behaviors is derived from laboratory research investigating nonhuman mammals. Laboratory rats have traditionally been the model of choice, but other rodents (mice, voles, gerbils, and hamsters) as well as sheep, rabbits and monkeys have been invaluable additional models that provide essential comparative information. Because at least some of the sensory, motivational, and motor aspects of maternal behaviors are taxonomically conserved, converging evidence gathered across species has outlined a core neural network for mothering. Recent functional magnetic resonance imaging (fMRI) studies in humans have confirmed and extended much of what we originally gleaned from research on nonhuman animals. Studies on this topic in the recent past have often separately considered the functions of a handful of brain structures commonly associated with regulating maternal behavior. However, it needs to be emphasized that such structures belong to interacting neural networks that integrate many processes contributing to successful mothering. These include neurobiological systems involved in sensorimotor integration, motivation, arousal, emotional processing, attentional selection, decision making, learning and memory, and inhibitory control. Together, they orchestrate the effective expression of behaviors appropriate to the needs of both mother and young.

Conceptualizations of the neurobiological underpinning of parental behaviors originally involved a single system that required a particular threshold of

hormonal and/or sensory stimulation from neonates to be reached before an appropriate caregiving response could be triggered.[416] Early detailed analyses of the behavior shown by female rats toward pups were not completely consistent with this conceptualization, though. Particularly inconsistent was that inexperienced nulliparous rats exposed to pups in a maternal sensitization paradigm are not simply neutral toward young and then spontaneously attracted to them a few days later, but instead exhibit what appear to be fearful responses and will actively avoid the offspring. As days of pup exposure progress, these females approach and investigate the pups more often without recoiling, tolerate physical contact from and even lick the pups, and then eventually seek them out. This can easily be contrasted with the wholly positive responses to pups displayed by almost all naturally parturient mothers.

These observations led to an alternative "approach–avoidance model" of mothering.[56,416] This model could be applied to any behavior involving a conflict between competing motivations and a shift in attraction to a social or even presumably nonsocial stimulus. Within this framework, the hormonal events of late pregnancy and parturition do act on the brain and peripheral sensory structures to shift the valence of pup sensory cues from negative to positive, but instead two distinct neurobiological systems are simultaneously modified for this to occur—an excitatory network promoting approach to and interaction with neonates, and an inhibitory system that promotes avoidance and withdrawal from them.[4] The balance of activity in these networks resolves the conflict between avoidance and attraction to pups such that mothering can be expressed. As discussed in detail below, in an attempt to match this model to the known neural systems regulating maternal behavior, the mPOA and adjacent ventral bed nucleus of the stria terminalis (BSTv) have been considered the primary "excitatory" nuclei that project facilitatory information to the mesolimbic dopamine system for the execution of maternal behavior. The MeA and its connections with the dorsal hypothalamus–anterior hypothalamic area (DH/AHA) and ventromedial nucleus (VMN) of the hypothalamus have been suggested to be the primary "inhibitory" nuclei with respect to maternal behavior.[4] In accordance with this model, the onset of maternal behavior is facilitated by a switch from functional dominance of the inhibitory MeA-to-DH/AHA–VMN system to the excitatory mPOA–BSTv-to-mesolimbic system.

It will be seen that the approach–avoidance model has been a very useful heuristic for understanding the neurobiology of maternal behavior, but there are caveats that must first be mentioned. Most problematic is that some brain sites can promote as well as inhibit the behavior, depending on the subpopulation of cells involved or the context in which the behavior is being studied. Thus, it is too simplistic to suggest that some neural sites or systems always promote maternal behavior while others inhibit such responses. Also, the evidence supporting this approach–avoidance model of mothering is heavily based on the inexperienced laboratory rat, in which avoidance of pups is very high and is alleviated by anosmia or sometimes continual exposure to their sensory cues. The model is not readily applied to other animals where parental behavior is spontaneously expressed at very high levels even by inexperienced members—they show no avoidance that needs to be overcome.

Importantly, there is probably also no such avoidance that needs to be overcome in many mammals living in their natural environments. Many animals live in large social groups containing numerous reproducing females. Juveniles in such groups are usually extremely interested in neonates and often have the opportunity to interact with young siblings or nonsibling conspecifics.[350] Similarly, in species like rats and other rodents with a postpartum estrus, lactating females living in natural environments commonly give birth to a subsequent litter before the older litter has reached the age of dispersal from the nest.[54] In either case, juveniles gain parental experience that may permanently obviate most of the aversion to offspring because this experience produces long-term effects on the brain that enhance later parental responding.[494–497] This early experience can affect later behavior to the point that, in its absence, some primate mothers are much more likely to kill or neglect their young.[498] Even in humans, previous experience with infants is a strong predictor of a new mother's positive responses to her own infant and feelings of maternal competency.[499–501]

Medial Preoptic Area (mPOA)

The brain site most studied for an involvement in parenting behaviors in mammals is the mPOA of the ventral forebrain, which lies just rostral to the anterior hypothalamus and caudal to the diagonal band of Broca. As mentioned in the section Components of Parental Care, it has been known that the mPOA was involved in parental behaviors since Fisher's work on testosterone-infused male rats in 1956.[44] A tremendous number of later studies from numerous laboratories have greatly expanded upon these findings by showing that a functional mPOA is essential for both the onset and early expression of maternal behavior. Premating, preparturitional, pre–Caesarean delivery, or postpartum destruction of the mPOA with electrolytic or excitotoxic lesions severely disrupts many components of maternal behavior in female rats, especially retrieval of pups and nest building.[3,4] A similar effect is produced by severing the dorsolateral axons coming to and emanating from the mPOA,[502–504] or by pharmacologically inactivating

the mPOA.[505,506] Licking the pups has not always been quantified in these studies, but when it is, it appears to be less negatively affected by mPOA lesions or knife cuts than other oral maternal behaviors.[504,507–509] The disrupted maternal behavior after mPOA lesioning is not due to endocrine disruption because such lesions impair the onset and expression of sensitized mothering in female and even male rats, whose maternal-like behavior is presumably independent of hormones.[3,4] Electrically stimulating the mPOA promotes maternal responsiveness in maternally experienced rats and in virgin female rats,[510] further supporting the idea that activity in the mPOA promotes mothering. Importantly, the behavioral effects of depressed mPOA activity are relatively specific, since it does not affect females' locomotor activity, sexual behavior, ingestive behavior, and ability to carry pup-sized inanimate objects around their home cage. The importance of the mPOA for many components of maternal behavior is conserved across species and these include laboratory mice,[511] California mice,[509] hamsters,[512] rabbits,[110] and sheep.[513]

Most studies agree that interfering with the mPOA severely to completely disrupts the active oral components of maternal behavior, but only moderate or mild alterations are found in nursing behaviors.[3,4] As discussed in the section Components of Parental Care, when deficits in nursing are found after mPOA manipulations, they may be secondary to the deficit in retrieval and the associated absence of a sufficient number of pups in the nest that can stimulate nursing. It is noteworthy in this context that mother rats with mPOA lesions or dorsolaterally severed axons approach and sniff pups in a manner similar to controls,[502,504,507,514] indicating that the mothers remain interested in pups and initiate physical contact with them. Additional lines of research further support the notion that the mPOA is involved in motivational processes associated with mothering. For instance, in an operant procedure, bar pressing for access to pups in early postpartum females is substantially reduced after mPOA lesions, whereas food-reinforced bar pressing is unimpaired.[508] Similarly, transient inactivation of the mPOA completely abolishes conditioned place preferences to pup-associated incentives.[515]

Relevant to understanding the role of the mPOA, or any other component of the brain's maternal behavior network, is that mammalian maternal care is provided over a long period of time lasting from weeks to years depending on the species. Across this period, the mother's responsiveness to her growing and maturing young changes to match their evolving needs. The temporal dynamics of mother–infant interactions have been described for numerous mammalian species,[99,100] and it is typical for mothers to relax their care in accordance with the increasing physiological and behavioral independence of the young. Indeed, as weaning approaches, mothers leave older young unattended more often and at greater distances, will reject their attempts for physical contact and nursing, and no longer restrain them from leaving the nest. Recent research has revealed that a substantial functional reorganization of the maternal circuitry occurs, probably sculpted by the continuous experience of interaction with the developing pups, to allow such changes in the mother's behavior across the postpartum period that are attuned to the needs of her young.

Specifically, Pereira and Morrell revealed that the mPOA is differentially engaged throughout the postpartum period to orchestrate maternal responses to the changing needs of the developing pups, from a necessary facilitatory role during early postpartum period to an inhibitory role during the late postpartum period.[506,516] Transient inactivation of the mPOA with the anesthetic bupivacaine during the first week postpartum was found to produce the expected loss of maternal responding in rats (Figure 51.14). The same mPOA inactivation during late lactation did not, and instead it increased maternal responding during this time when responsiveness has waned (Figure 51.14).[506,516] Fascinatingly, such changes in the mPOA could be occurring simultaneously with the demonstration of the full pattern of maternal behavior toward a new litter of pups born as a result of mating during the postpartum estrus,[15,496] and may involve even a different pattern of activity in the mPOA and elsewhere to allow dams to both ignore the older litter while remaining interested in the recently born pups. There is also some evidence from immediate-early gene studies that some cells in the mPOA and also the BSTv respond to the aversive qualities of pups, possibly inhibiting maternal behavior, because nonmaternal nulliparous rats have almost as much Fos expression in the mPOA–BSTv after forced exposure to pups as do maternal rats in some studies.[517]

Another consideration for a more comprehensive view of the mPOA in mothering is that long after weaning of the litter, the female brain remains primed for future maternal responding, and experienced females are more resistant to sensory and neural insults compared to inexperienced females.[418,508,513,518] Even nulliparous sensitized female rats are more likely to act maternally at a future date than inexperienced females, indicating that experiential effects do not require endocrine factors.[519,520] The neural basis of this may be related to the finding that second-time mothers have more glial fibrillary acidic protein (GFAP)-immunoreactive cells (i.e., astrocytes) in the mPOA compared to virgins or first-time mothers, and this parity difference requires that females maternally interact with pups after parturition.[521] The functional implications of an increased number of astrocytes are unknown, but perhaps they contribute to a mother's long-term interest in pups by modulating the adjacent

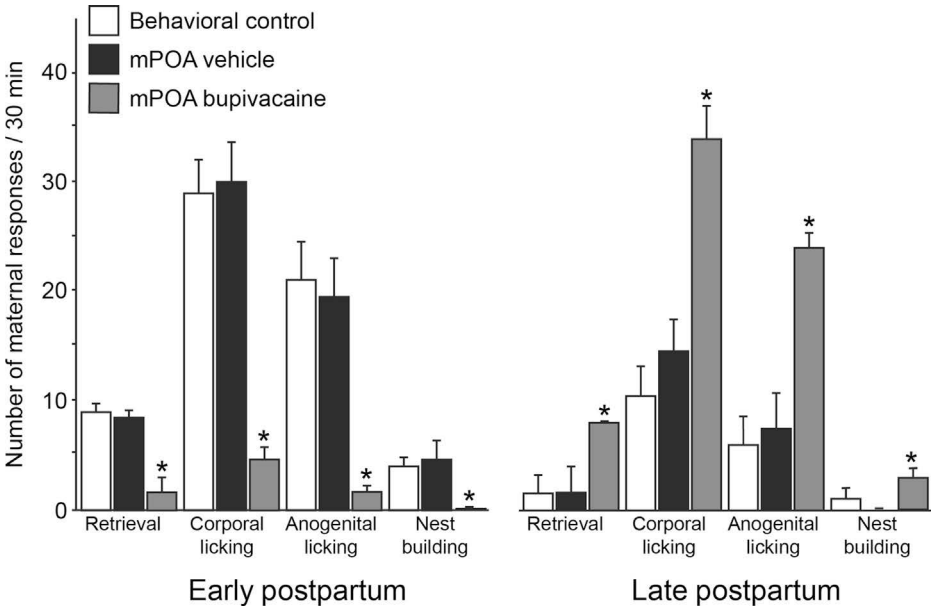


FIGURE 51.14 Frequencies of maternal behaviors (Median ± SIQR) shown by female rats tested early postpartum (days 7 and 8) or late postpartum (days 13 and 14) after transient inactivation of the mPOA with 2% bupivacaine or no inactivation by infusing saline. The behavioral control group received no stereotaxic surgery or infusion. Inactivating the mPOA disrupted all measures of active maternal behavior when performed early postpartum, but facilitated the same behaviors when performed late postpartum, indicating the differing role of the mPOA for maternal behavior across the postpartum period. *Source: Modified from Pereira and Morrell, 2009.*[506]

preoptic area neurons necessary for maternal motivation or behavior. Other types of structural plasticity in the mPOA, such as changes in the size of its somata and the number of basal dendritic branches, occur across pregnancy and in response to exogenous ovarian hormones but are not permanent,[522,523] so they are more likely involved in the onset of maternal behavior rather than long-term experience effects.

Numerous neurotransmitters are released in the intact mPOA to promote or inhibit maternal behaviors. Compared to nonmaternal nulliparous rats, mother rats have greater dopaminergic and serotonergic activity in the mPOA,[524,525] and when they interact with their offspring, AVP is locally released at high levels.[526] In ewes, the release of dopamine, gamma aminobutyric acid (GABA), and OT increases in the mPOA during interaction with the lamb, but there is no change in serotonin release.[227] Infusion of pharmacological agents that mimic or block the activity of a number of neurochemicals alters maternal behaviors. For example, various aspects of maternal behavior are disrupted by mPOA administration of a $GABA_A$ or $GABA_B$ receptor agonist,[505] D1 or D2 receptor antagonists or a dopamine reuptake inhibitor,[527–530] α2-autoreceptor antagonists that increase norepinephrine release,[531] and a nitric oxide synthesis inhibitor that could blunt the release of a number of neurotransmitters.[532] Serotoninergic signaling is essential for maternal behavior in rodents,[533,534] but the effect of serotonin receptor modulation specifically in the mPOA has not yet been investigated.

While most of the research on the mPOA shows little effect on nursing, does the mPOA really have nothing to do with this essential component of mammalian maternal behavior? A subset (~5%) of mPOA cells that express Fos during maternal behavior project to the midbrain periaqueductal gray (PAG),[535] and this projection has been proposed to regulate nursing behavior in postpartum rats.[536] Neurons in the ventrocaudal PAG uniquely show very high Fos expression in response to suckling inputs from pups,[537,538] and lesioning this site greatly interferes with kyphosis while leaving nursing in other postures and appetitive maternal behaviors such as retrieval intact.[537,539] Kyphosis is tonically inhibited by GABA release in the ventrocaudal PAG, but the posture is disinhibited by suckling inputs impinging on PAG cells that are involved in postural control.[540] Because dams cannot simultaneously show immobile nursing and active maternal behaviors, it has been proposed that inhibitory reciprocal projections between the mPOA and ventrocaudal PAG ensure the suppression of inappropriate maternal responses depending on the presence or absence of pup suckling.[537]

Immediate-Early Gene Expression

Studies using expression of the immediate-early genes *c-fos*, *FosB*, and *Egr-1* as markers for cellular modulation in response to cues from pups or behavioral interactions with them have helped confirm and further explicate the role of the mPOA in mothering behaviors. Very large increases in immediate early-gene expression are found in the mPOA when sensitized virgin or postpartum female rats interact with pups,[443,541–545] and this increased expression can persist for many hours (Figure 51.15).[546] If postpartum females are only distally exposed to pups at testing, however, Fos is not increased in the mPOA compared to unexposed controls.[443,542] The same is true for virgin and postpartum laboratory mice,[547,548] but postpartum rabbits require only distal

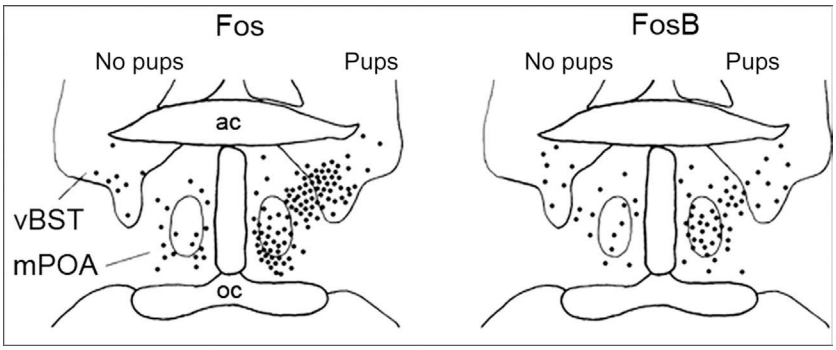


FIGURE 51.15 Schematic representation of frontal sections through the postpartum rat medial preoptic area (mPOA) and ventral bed nucleus of the stria terminalis (BSTv) showing the location of Fos-immunoreactive (left) and FosB-immunoreactive (right) cells after no interaction with pups (left half of each panel) and after an interaction with pups and the display of maternal behavior (right half of each panel). Ac: anterior commissure; oc: optic chiasm. *Source: Modified from Numan et al., 2006.*[4]

cues from pups for peak Fos expression.[549] The importance of the behavioral interaction with offspring rather than their distal cues in rats is further supported by the inability of olfactory bulbectomy, peripheral anosmia, thelectomy, or general ventral anesthesia to reduce Fos-immunoreactive cells in the mPOA as long as the females behave maternally.[443,444,544] Nonetheless, the development of fMRI for use in live rodents has revealed that even when postpartum rats are restrained and cannot actively interact with pups, the maternal mPOA still exhibits positive blood oxygenation level–dependent activation (often thought to reflect the increased blood flow and metabolism associated with elevated cellular activity) in response to pups' ventral probing and suckling.[550] In multiparous sheep, the vaginocervical stimulation associated with parturition induces Fos in the mPOA, but Fos is not further increased by the presence of the lamb.[551,552] This could indicate little involvement of the mPOA in response to lamb cues or the expression of maternal behavior soon after parturition, but a more reasonable interpretation is that parturition generates a ceiling effect in Fos-immunoreactive cells in the ewes' mPOA. This is reasonable because inactivation of the mPOA during parturition with the anesthetic lidocaine does prevent the onset of maternal behavior in primiparous ewes. Lidocaine in ewes' mPOA also reduces some aspects of ongoing mothering when infusions begin hours after parturition.[513]

This immediate-early gene activity in the maternal mPOA is a convenient marker for genomic activity in response to offspring cues or the expression of parenting, but the specific function of these Fos-immunoreactive cells in the mPOA remains an important question. Half to 75% of the mPOA cells expressing Fos after postpartum rats and mice interact with pups also express glutamate decarboxylase 1 (GAD_{67}), so these cells are likely GABAergic.[511,553] Activation of so many potentially inhibitory cells in the maternal mPOA could reflect the removal of tonic inhibition on downstream cell groups necessary for maternal behavior, or that activation of these GABAergic cells is suppressing other cell groups that would otherwise interfere with the behavior.[553] A recent anatomically refined study found that the subregional location of the densest Fos in the mPOA after the display of maternal behavior by postpartum mice was not the same mPOA subregion where maternal behaviors were the most disrupted by excitotoxic lesions, suggesting that many of the mPOA cells are responding to maternal interaction with pups for some other behavioral or physiological purpose.[511] A major limitation of these immediate-early gene studies is that this approach has little temporal resolution. Progress in understanding maternal behavior is hindered by the lack of understanding of the real-time events and the coordinated and sequenced activity across neural components necessary for mothering. Particularly useful would be functional circuit analysis with electrophysiological approaches and continued use of fMRI in mother laboratory rats.

Some insight into the role of the immediate-early genes themselves in maternal behavior, other than just being convenient markers for cellular modulation, comes from virgin female mice with a targeted deletion of the *FosB* gene. These mice are less likely to spontaneously retrieve pups as virgins, and even after mating and giving birth to healthy pups still will not care for them; most pups die within days after birth.[554,555] Inhibiting phosphorylation of the extracellular signal-regulated kinase protein (ERK), which is part of the intracellular cascade triggered by growth factors and necessary for *FosB* transcription, also inhibits spontaneous retrieval in inexperienced virgin female mice, but does not do so in experienced postpartum mothers.[556] Because *FosB* knockout mice are hyperactive, impulsive, and possibly hyperemotional, and their brains contain markers of neuropathology, the relationship between *FosB* and maternal behavior may be nonspecific.[555]

Relationship of the mPOA with the BSTv

The cells relevant for maternal behavior extend out of the cytoarchitectorally defined borders of the mPOA and into the adjacent BSTv (including parts of the dorsomedial, dorsolateral, magnocellular, and anteroventral subnuclei of the BST). Because of the close proximity of

the two sites, many mPOA lesion and hormone infusion studies have probably also affected cells of the BSTv,[557] and the mPOA and BSTv have sometimes been presented as a common functional unit for maternal behavior because distinguishing their individual roles is difficult.[3,557] Nonetheless, it has been observed that whereas retrieval does not recover after mPOA lesions,[514,558] it does reemerge in some postpartum rats with neurotoxic lesions of the BSTv.[557] Similarly, in primiparous sheep, the onset of maternal behavior at parturition is almost completely prevented by temporary inactivation of the mPOA, while almost no deficits are seen after BSTv inactivation.[513] Differences between the function of the mPOA and BSTv are also indicated by the findings that retrieval is more greatly impaired after mPOA than BSTv infusion of idazoxan (an α2-autoreceptor antagonist that increases norepinephrine release)[531] and that dampening AVP V1a receptor activity in the mPOA reduces retrieval and nursing in postpartum rats while having no effect in the BSTv.[526,559]

These results distinguishing between the two sites are probably related to the different projections of mPOA and BSTv cells that are stimulated during the performance of maternal behavior. The termination sites of almost half of all Fos-expressing neurons in the mPOA and BSTv of maternally acting female rats have been identified and involve just six brain areas—the lateral septum, ventromedial hypothalamus, lateral habenula, retrorubral field, ventral tegmental area (VTA), and lateral PAG. While most of the identified mPOA Fos-containing cells projected to the two forebrain sites (the ventromedial hypothalamus and lateral septum), the identified BSTv Fos-containing cells projected more widely to the six targets.[535] This functional and anatomical information could suggest that mPOA output primarily modulates activity in brain sites that would otherwise inhibit maternal behaviors, including the ventromedial hypothalamus and lateral septum, whereas the BSTv functions more to activate midbrain motor and motivation systems necessary for maternal behaviors to be displayed.

mPOA as a Site for Hormone-Sensory Integration

The mPOA is an essential component of the maternal behavior network, in part because it is perfectly situated to integrate an animals' endocrine state with incoming sensory cues from young, and then transmit this information to motivation and motor effector pathways that orchestrate the expression of maternal behaviors. Indeed, the mPOA is exquisitely sensitive to ovarian and pituitary hormones and a primary neural site for their effects on the onset of maternal behavior. It contains very high densities of receptors for ER, PR, PRL, and OT, all of which fluctuate in their expression near the end of pregnancy and in the early postpartum period in many animals.[110,131,180,560–562] When estradiol is implanted or repeatedly infused into the mPOA of nulliparous rats, the onset of retrieving is rapid, although other maternal behaviors are not necessarily so quickly established.[205,563,564] In rabbits, which do not retrieve young, estradiol implanted into or very near the mPOA stimulates components of maternal nest-building behavior.[565]

Even though estradiol in the mPOA stimulates components of maternal behavior in virgin animals, implanting the selective ER modulator tamoxifen (often traditionally used as an estrogen receptor antagonist in the brain) into the mPOA 2 days before parturition does not prevent the onset of maternal behavior.[566] Tamoxifen implants into the mPOA do somewhat reduce the percentage of females showing maternal care (by ~33%) if litters are delivered by Caesarian section,[566] suggesting that the experience of parturition and release of its hormones may override the need for late-pregnancy estrogenic activity in the mPOA. Recently, this question was re-examined by studying the maternal behavior of female laboratory mice after mPOA delivery of a short hairpin RNA (shRNA) that interferes with the mRNA for ERα. When testing began 4 days after parturition, these females displayed rather severe deficits in almost all maternal behaviors.[141] Unfortunately, it remains unknown how soon after parturition maternal behavior began to be impaired, if these mothers were lactating normally, or what the mothers were doing in the absence of spending time with the litter. Once ER signaling in the mPOA helps establish maternal behavior, it may continue to help maintain the behavior even in the absence of estrogen. A large percentage (~33%) of the mPOA cells expressing Fos during the display of postpartum maternal behavior also contain ERα,[567] and in the absence of high levels of circulating estrogen during lactation, activation of ERα in these cells may instead occur through a ligand-independent mechanism involving stimulation of the receptors by classic neurotransmitters.[568]

The sites where PRL acts to hasten maternal behavior are certainly within the central nervous system, as daily intracerebroventricular infusion of PRL or placental lactogens facilitated the behavior in steroid-primed females.[188] Bakowska and Morrell presented similar conclusions from pregnant female rats that were hysterectomized and ovariectomized on day 16 of pregnancy.[569] Conversely, a PRL receptor antagonist delivered into the cerebral ventricles delays the onset of mothering in parturient rats.[570] The mPOA is one site mediating these effects. Similar to ventricular infusion, PRL or placental lactogens infused into the mPOA can act along with ovarian hormones to hasten the onset of maternal behavior in nulliparous rats;[188,571] antagonism of PRL receptors in the mPOA delays the onset of

mothering in nulliparous steroid-treated rats.[572] Other brain sites where PRL acts to promote maternal behavior are unknown.

The mPOA is also a target for the effects of OT and its receptor antagonist on maternal behavior,[207] and fMRI activity in the POA is blunted by pretreating mother rats with an OT receptor antagonist before exposing them to pups.[550] Surprisingly, OT release in the mPOA does not increase during mother–infant interactions,[526] even though OT acts there to promote maternal behavior and individual differences in OT receptor expression in the mPOA are associated with the frequency of postpartum maternal licking.[208] Other forebrain sites where OT promotes mothering are mostly unknown, but maternal behavior can be stimulated by infusing OT into the olfactory bulb[573] and impaired by OT receptor antagonism in the VTA.[207] It was noted in the section Hormones Most Significant for the Onset of Maternal Behavior, that reducing OT signaling to the mPOA and elsewhere by lesioning the PVN of the hypothalamus, the major source of OTergic projections in the brain, disrupts the onset of maternal behavior at parturition but has little effect on its maintenance.[198,199,574] Other neuropeptides or their receptor antagonists that can influence maternal behavior after mPOA infusion include AVP,[207,559] endogenous opioids,[575] cholecystokinin,[576] and melanin-concentrating hormone.[577]

Progesterone is a strong inhibitor of maternal behavior (see the section Hormones Most Significant for the Onset of Maternal Behavior), and the mPOA has been studied as its target for this effect, but hysterectomized and ovariectomized pregnant female rats with progesterone implanted in the mPOA at the time of surgery are still rapidly maternal.[578] Perhaps progesterone acts elsewhere or on multiple sites simultaneously to produce its inhibitory effects on maternal behavior, but Sheehan and Numan did find that progesterone prevented the increase in Fos expression in the mPOA (and BSTv) of hysterectomized and ovariectomized pregnant female rats given an injection of estradiol that would have rapidly stimulated maternal behavior.[579] Circulating progesterone levels begin to rise a few days after parturition and remain very high through the second week of lactation in rats (see the section Hormones Most Significant for the Onset of Maternal Behavior), but it is unknown why this endogenous progesterone acting in the mPOA or elsewhere does not interfere with the maintenance of maternal behavior. Maybe this can be avoided because basal progesterone receptor expression is very low in the postpartum mPOA,[560,580] rendering it insensitive to progesterone's potential inhibitory effects on mothering.

With regard to sensory inputs from pups, the mPOA receives converging information from virtually all sensory modalities and sends input back to those sources. Olfactory information from the main and accessory olfactory bulbs reaches the mPOA via the MeA,[581,582] and the mPOA receives ventral trunk somatosensory inputs directly from the peripeduncular nucleus of the lateral midbrain[582] and perioral tactile inputs through a direct trigeminohypothalamic projection[583] and indirectly via brainstem relay nuclei.[582] Gustatory input can reach the BST,[584] which, in turn, is densely interconnected with the mPOA. Moreover, olfactory, gustatory, somatosensory, auditory, and visual regions within the insular cortex project to several limbic and sensorimotor regions and densely to the infralimbic region of the medial prefrontal cortex (mPFC), which, in turn, also projects to the mPOA.[582]

Connections of the mPOA for the Elicitation of Maternal Behaviors

Motivation Systems

Primary efferents from the mPOA traveling through the lateral hypothalamus allow it to interact with components of the mesolimbic dopamine (DA) system for the motivational aspects of maternal responsiveness to pups. The mesolimbic DA system, which is composed of DA cell bodies in the midbrain VTA that project to the nucleus accumbens (NA) in the forebrain, has been recognized for its central role in several behavioral functions related to motivation.[585,586] The NA receives converging excitatory inputs from most cortical and limbic structures, and hence has long been considered an interface linking those corticolimbic structures to behavioral output systems, under the modulatory influence of DAergic inputs from the VTA.[587] Neurophysiological and neuroanatomical studies suggest that DA in the NA may select and integrate the effects of limbic and cortical afferents, thus influencing the transmission of information to output areas, and ultimately modulating goal-directed behaviors.[586,588]

Anatomically and functionally, the NA can be divided into two distinct regions, the core and the shell.[589,590] Each receives different but overlapping excitatory glutamatergic projections from corticolimbic structures, including the basolateral amygdala (BLA), hippocampus, and prefrontal cortex (PFC).[589,591] The core and shell also differ in their downstream targets. The NA core projects primarily to the dorsolateral portion of the ventral pallidum, the substantia nigra pars reticulata, and the subthalamic nucleus.[590,592] Projections from these targets travel to the motor thalamus and then to cortical motor areas for the execution of behavior. The NA shell, in contrast, sends projections mainly to the ventromedial ventral pallidum, substantia innominata, mPOA, several hypothalamic nuclei, substantia nigra pars compacta, VTA, and PAG.[592–595]

In the context of maternal behavior, several pieces of evidence indicate that DA released in the NA shell and core

participates in activating maternal behavior. For instance, many laboratories have used microdialysis to find that DA release in both the core and shell of postpartum mother rats is enhanced during interactions with pups.[596–599] It appears that the hormones associated with pregnancy and parturition bias the mesolimbic DA system to respond to pups by reducing mother's basal DA levels in the NA shell. As a result, the magnitude of pup-evoked DA release is more striking.[596,600] In a very recent study, DA responses in the NA were further characterized during a fine-grained analysis of mother–pup interactions across the postpartum period. It was found that during the early postpartum period, the presentation of pups behind a screen that elicited maternal pup-seeking behavior resulted in a robust increase in DA release in the NA core, which was further augmented during active but not passive maternal interaction with pups. Although this pattern of DA release was also found in late-postpartum females, the magnitude of release was considerably attenuated in those mothers.[601]

Finer temporal analysis using in vivo voltammetry has also revealed that the DA signaling in the NA shell and core increases with the initial presentation of pups after a brief separation, and also immediately before as well as during active maternal behaviors such as pup retrieval and licking (Figure 51.16).[597,602] Furthermore, the magnitude of DA release is related to individual variation in maternal licking, such that high-licking mothers show a greater increase in DA release before or during a licking bout compared to low-licking mothers.[597] Levels of DA receptor expression in the NA are also positively correlated, and DA transporter levels negatively correlated, with mothers' levels of licking.[597] Together, these studies suggest that changes in NA DA activity associated with mother–pup interactions may serve as a neural substrate for the dynamic motivational aspects of maternal behavior among individuals and across postpartum time.

Interfering with this DA neurotransmission by electrolytic or DA-depleting lesions of the VTA, pharmacological manipulation of VTA activity, or core or shell administration of D1 or D2 receptor antagonists each selectively and severely disrupt most active maternal behaviors in early-postpartum rats.[529,603–609] The amount of time these mothers spend with the pups is normal, indicating that they remain interested in approaching the pups and maintaining physical contact with them. Indeed, these VTA or NA manipulations can even facilitate nursing behavior if pups are manually placed in the nest,[536] which is consistent with studies showing that manipulating accumbens DA selectively influences

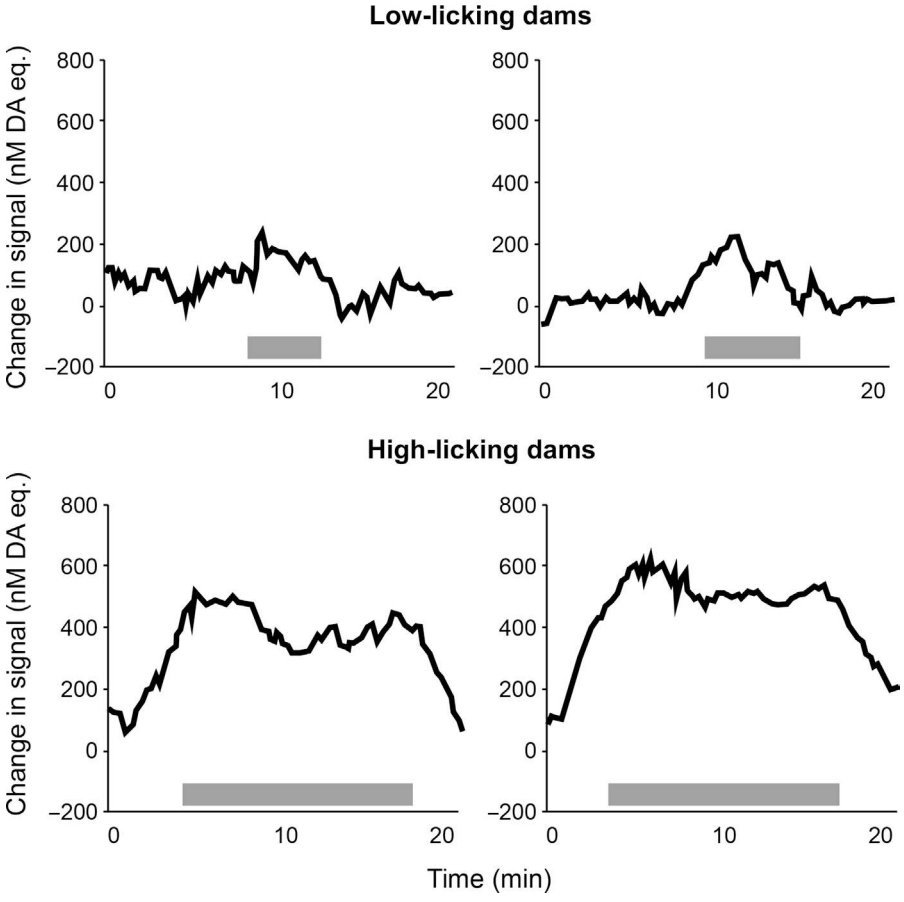


FIGURE 51.16 Changes in dopamine (DA) signaling in the shell of the nucleus accumbens of two representative high-licking and low-licking postpartum rats before, during, and after a bout of licking their pups. The gray bar indicates the duration of the licking bout. Note the greater increase in high-licking rats and that the rise in DA signaling begins to occur before licking is exhibited. *Source: Modified from Champagne et al., 2004.*[597]

the activational or effort-related aspects of motivated behaviors, while leaving the directional aspects relatively intact.[586,610] The disruptive effects of DA receptor antagonism on active maternal behaviors are not exclusive to rats and occur in both sexes of the biparental prairie vole.[611] Consistent with these studies, D1 receptor stimulation of the NA promotes the onset of maternal behavior in hysterectomized, pregnant female rats.[530] Of course, DA in the NA does not activate maternal behaviors in chemical isolation and the purine nucleoside, adenosine, and the adenosine A_{2A} receptor have been found to modulate DA-mediated maternal behavior.[612]

The nature of the impairments seen after interfering with DAergic activity is thought to mostly reflect deficits in maternal motivation, which is demonstrated by the fact that the deficits can be overridden if dams are separated from pups for 3–12 h[605,613] or if the motivational salience of the pups is increased by presenting mothers with hungry, demanding pups.[614] In both cases, active components of maternal behavior are restored to levels characteristic of control dams. Also, if postpartum rats are muzzled so they cannot retrieve pups, they spend considerable time pushing at the pups with their snouts and handling them with their paws, but such compensatory behaviors are not displayed if mesolimbic DA transmission is reduced.[615] Furthermore, DA release in the NA contributes to the rewarding aspect of the pups, as mothers can form a conditioned place preference for an environment in which they interacted with pups, but not if DA release in the NA is impaired.[599,607,616]

Given the results of the studies described immediately above, it is probably surprising to read that lesioning the NA produces no deleterious effects on maternal behaviors in rats.[508,617] To reconcile this, it is essential to consider prevailing models of how the NA is involved in basal ganglia function. These models propose that the NA consists of distinct populations of medium spiny GABAergic projection neurons that selectively express either D1 or D2 receptors.[618] These neurons respectively project directly or indirectly to basal ganglia output nuclei, namely, the substantia nigra pars reticulata and pedunculopontine tegmental nucleus.[619,620] Through these opposing direct and indirect output pathways, NA neurons are proposed to select appropriate behavioral responses while inhibiting competing ones. Manipulations that interfere with DA neurotransmission in the NA (inactivating the VTA, intra-NA DA receptor antagonism, and depletion of DA within the NA) all result in a marked impairment in behavioral responding to pups and related stimuli. By contrast, transient inactivation or permanent cell body lesions of the NA uniformly inhibit all its neurons, regardless of the medium spiny neuron subtypes or their efferent destination, and have virtually no effect on motivated behaviors. Given the distinct populations of medium spiny neurons within the NA, eliminating all NA activity might produce effects that negate each other at the level of basal ganglia output, with no net change in behavior.

In an elegant series of studies, Numan and collaborators have provided substantial evidence supporting the idea that activation of hormonally primed and offspring-stimulated mPOA projections to the VTA and NA promotes maternal behavior.[621] The mPOA can influence NA function through a direct projection to it and indirectly through projections to the VTA.[622,623] The mPOA projections to DAergic neurons in the VTA include OT-expressing cells,[624] providing a neuroanatomical substrate for how OT modulates maternal behavior.[207] Moreover, infusion of OT into the VTA can trigger DA release into the NA,[625] and this promotes the onset of maternal behavior and probably influences its ongoing display.[207] Intact bilateral connections between the mPOA and the VTA and NA are essential for maternal behavior in rats. In a study using an asymmetrical lesion design,[606,626] unilateral mPOA lesion paired with a contralateral lesion of the VTA severely disrupted maternal retrieval of pups, while various control lesions were relatively ineffective. Consistent with these results, the NA shows increased Fos expression during the display of maternal behavior in rats,[542] but not if the ipsilateral mPOA is lesioned.[627]

After hormone and pup sensory information has reached the NA via the mPOA, and probably to a minor degree via the basolateral amygdala,[628,629,630] this information is transmitted to the ventral pallidum. The ventral pallidum is reciprocally connected via GABAergic neurons to the NA and responds to the pleasurable qualities of stimuli such as pups.[588] The ventral pallidum also projects to motor networks necessary for the execution of behavior.[631] Lesioning or infusing a $GABA_A$ receptor agonist into the ventral pallidum causes a dramatic disruption in maternal responsiveness,[558,617] as does a unilateral lesion of mPOA paired with a contralateral lesion of the ventral pallidum.[617] Together, these studies demonstrate the importance of the mPOA interactions with the VTA–NA–ventral pallidum circuitry in the activational aspects of maternal motivation and behavior.

Emotion Regulation Systems

The approach–avoidance model of maternal behavior in rats suggests that neophobia of pup-related cues is a primary impediment to maternal responding in inexperienced rats, theoretically by high neural activity in brain sites involved in aversion or negative emotional reactivity that do not allow the mPOA and its connection to the brain's maternal motivation systems to dominate.[416] Because the odors of pups have traditionally been thought to be the basis of this neophobia, the locus of inhibitory control of mothering has been on the MeA,

which receives projections from both the main and accessory olfactory bulbs.[581] The MeA very densely and reciprocally innervates the mPOA, and each site can inhibit or excite the other. Estradiol reduces excitatory input from the mPOA to the MeA, which may be the basis of how estradiol regulates this projection to facilitate mothering.[632]

Similar to the effects of anosmia, nulliparous or pregnancy-terminated female rats sustaining electrolytic or cell body–specific lesions of the MeA, and sometimes also the cortical amygdala, are faster to act maternally than unlesioned controls.[422,436,633] These lesioned females do not actively avoid pups placed in their nest and are more tolerant of contact with them even if not yet expressing maternal behavior.[422] An intact mPOA is required for this facilitation, suggesting that the absence of the MeA does not alone promote maternal behavior but instead removes an inhibitory input to sites that do.[634] The onset of maternal behavior in MeA-lesioned animals can still take a few days, so it is not as effective as peripheral anosmia, suggesting that some olfactory inputs inhibiting maternal behavior are transmitted elsewhere in the brain. As opposed to the positive effects of MeA lesions in nulliparous rats, repeated kindling-like electrical stimulation of the MeA inhibits the future resumption of mothering in experienced postpartum rats. Furthermore, MeA kindling also somewhat reduces the preference for pup-related cues by inexperienced nulliparous rats, but it does not further affect their already low maternal responding in a sensitization procedure.[510] Nulliparous ewes also find offspring olfactory cues aversive, but it remains unknown if deactivating the MeA elicits maternal behavior. It is known that temporary chemical inactivation of the MeA or nearby cortical amygdala (but not the basolateral amygdala) beginning at parturition does not affect the onset of maternal behavior, but it does render ewes non-selectively maternal when tested hours later. Later MeA inactivation in already-selective ewes does not produce this effect, indicating that the MeA must process cues necessary for establishing an olfactory memory of the lamb (Figure 51.17).[635]

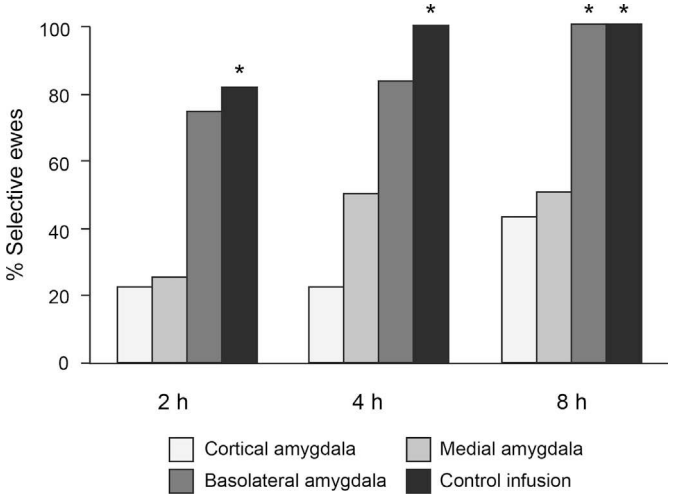


FIGURE 51.17 Percentage of ewes that selectively interacted with their lamb after 2, 4, or 8 h of inactivation of the cortical (Co), medial (Me), or basolateral (Bl) amygdala with lidocaine beginning at parturition. Inactivation of the cortical or medial nuclei, but not the basolateral nucleus, disrupted selectivity for the lamb. *Source: Modified from Keller et al., 2004.*[635]

Note that in animals requiring olfaction for the expression of maternal behavior, such as voles and mice, MeA lesions could be expected to produce blunted interest in pups, but this has not been examined in female voles, and lesion studies in mice have not targeted the MeA.[49,636,637] Another consideration regarding the MeA mentioned earlier in this chapter is that cell body–specific lesions of the MeA are incapable of promoting maternal responding in nulliparous female rats if their ovaries had been removed,[436] suggesting that the endocrine events involved with pseudopregnancy that can be induced after some types of MeA lesions are responsible for the effects on maternal behavior. In support, pharmacologically inhibiting pituitary PRL release prevents the facilitation of maternal behavior after excitotoxic MeA lesions. In addition, by delaying testing until well after postlesion pseudopregnancy has terminated, there is only a modest facilitation of maternal responding.[436] Such results might suggest that the negative influence of the MeA on maternal behavior in rats has been overemphasized, although it is interesting that in another series of studies using ovariectomized nulliparae tested 5 days after receiving electrolytic MeA lesions, there was still a dramatic onset of mothering compared to controls.[422] This apparent inconsistency could be related to differences between the studies in the neuroanatomical extent of the lesions or the lesion methods used (electrolytic versus excitotoxic).[638]

The MeA not only projects heavily to the mPOA but also provides major efferent projections to the DH/AHA and VMN of the hypothalamus, which are thought to partly mediate the MeA inhibition of maternal behavior. The DH/AHA and VMN are more generally components of the neural network mediating aversive and defensive behaviors,[639] and electrical stimulation of at least the VMN inhibits the firing of many mPOA neurons.[640] Aversive pup stimuli do not elicit Fos in the VMN of nonmaternal rats, but they do in the DH/AHA,[517] and MeA neurons that are activated in nonmaternal rats by aversive pup stimuli project to both hypothalamic regions.[633] Important direct evidence for the inhibitory role of these sites is that excitotoxic lesions of the DH/AHA or VMN stimulate maternal responding in steroid-primed nulliparous rats.[641] An indirect route through which AHA–VMN output may regulate avoidance of pups in nonmaternal rats is through projections to parts of the midbrain PAG involved in defensive responses. It would be valuable to

determine if lesioning any subregion of the PAG can alter defensive responding and promote maternal behavior in nulliparous rats. Ventrocaudal PAG lesions do reduce anxiety in postpartum rats,[74] and destruction of the rostral lateral PAG reorients the focus onto pups in morphine-treated postpartum rats that would rather hunt than mother.[642] Other brain sites, including the septum, central amygdala, mammillary region, and midbrain raphe serotonin system, could also interact with the MeA, DH/AHA, and VMN to instill aversion to pups in nonmaternal rats.[4,643] Of particular mention in this context is the lateral habenula, which regulates midbrain raphe cells to elicit or suppress aversive responses,[644] and is known to be necessary for the onset but not maintenance of natural or sensitized maternal behavior in rats.[645,646]

The function of the MeA has been suggested to switch after female rats give birth, by acting at that point to transmit olfactory inputs to the mPOA to increase attraction to pups and maternal behaviors.[4] There is little experimental support for this; on the contrary, olfaction is not needed for postpartum maternal behavior in rats (see the section Components of Parental Care), and increasing inhibitory tone in the MeA with the $GABA_A$ receptor agonist muscimol has no effect on postpartum mothering.[629]

The MeA regulates innate fear responses to olfactory stimuli,[647] which is consistent with the promotion of maternal responsiveness after MeA lesioning, but these lesions also reduce emotional reactivity unassociated with social odors. Female rats with corticomedial amygdala lesions not only are more maternal but also spend more time in the center of an open field, indicative of reduced general anxiety.[422] A regimen of exogenous ovarian hormones that presumably acts in part on the MeA to induce maternal behavior also increases time in the center of an open field.[441] Conversely, nulliparous female rats that received kindling-like electrical stimulation of the MeA spend less time in the center of the open field, indicative of higher anxiety.[510] The question arises whether MeA lesions promote maternal behavior by removing the ability to process aversive pup odors, or because of a more general dampening of emotional reactivity. It seems that for the onset of maternal behavior in laboratory rats, the olfactory modification is the most relevant. This is supported by data indicating that maternal interest increases in the final few days of pregnancy in female rats,[89] but their general anxiety-related behaviors in an open field or an elevated plus-maze are not lower than those of cycling females.[648,649] Second, if a general reduction in emotional reactivity was necessary for the onset of maternal behavior, treating rats with anxiolytics would be predicted to hasten maternal sensitization in nulliparous rats, but it does not.[650] Third, olfactory bulbectomy or peripheral anosmia very quickly induces maternal behavior, but does not in all studies reduce anxiety-related behaviors.[651,652] Lastly, nulliparous female rats that had juvenile experience with pups show very low adult anxiety-related behavior, meeting the low levels found in most postpartum rats. These adult nulliparae still avoid pups, though, and require a days of exposure to young before acting maternally, although they do sensitize faster than females not exposed to pups during juvenile life.[497]

A generalized reduction in anxiety or other aspects of emotional reactivity may not critically contribute to the natural onset of maternal behavior in laboratory rats, but there is such a reduction in anxiety after females have given birth. Lower anxiety behavior can be found in many behavioral paradigms within 24 h after parturition, lasts for about 1 week postpartum, and requires recent physical contact with the litter but not their suckling.[50] This mitigated reactivity to anxiogenic stimuli in maternal animals that have recent litter contact is reflected by lower Fos expression in some brain areas traditionally associated with emotional regulation.[653] The effectiveness of mother–offspring touch to suppress anxiety or other negative affective behaviors is further seen by the blunted reactivity of nulliparous, maternally sensitized female rats.[654–656] This blunted anxiety accompanying motherhood in rats also occurs in women,[50] but mother rhesus monkeys appear to be more anxious when they have infants to care for.[657]

As discussed elsewhere,[3,50] this general reduction in anxiety may affect numerous other postpartum behaviors. A suppression of anxiety could reduce dams' reactivity to intruders to the nest site in a way that is permissive for maternal aggression, although some data do not support this.[52] Low anxiety may also help maintain maternal attention to pups under mildly threatening conditions, but this suggestion is complicated by the findings that mother rats genetically selected for high anxiety spend more time with pups under undisturbed conditions and are faster to retrieve them under either undisturbed or challenging conditions compared to low-anxiety dams.[658] Others have found no relationship between "trait" anxiety in female laboratory mice and their later maternal behavior.[659] It has also been reported that noradrenergic manipulations in the BSTv, long associated with anxiety-related behaviors in addition to maternal behaviors, can impair mothering but not necessarily alter anxiety in postpartum rats.[531] It may be the case that within a natural environment, low postpartum anxiety is most important for achieving the metabolic demands of lactation, by allowing dams to forage further from the nest and take greater risks to obtain food. The more time that the dam spends away from the nest would lead to a gradual loss of the sensory "trace" instilled by pups that is needed for her low anxiety, and the resultant rise in anxiety could drive her to return to the familiar environs of the nest where she would then reunite with the pups.[660]

Interestingly, some of the emotional consequences of motherhood persist long after lactation, and anxiety can be lower in mothers tested many months after weaning of the litter.[661,662] This depends on when previous mothers are tested because cycling, primiparous female rats are less anxious than nulliparous females during the afternoon of proestrus but not during other phases of the estrous cycle.[662] This suppression of anxiety is clearly not due to recent contact with pups but instead due to the enduring neuroendocrine effects of parity, including lower PRL and estrogen release in previous mothers compared to nonmothers, but enhanced sensitivity to estradiol from greater ERα expression in the pituitary gland and brain—including in the mPOA.[663]

Cognitive Systems

Maternal behaviors emerge once the mPOA–mesolimbic pathway is activated and the MeA–DH/AHA–VMN inhibitory pathway is deactivated, but caregiving and its associated behaviors can be further improved by fine-tuning other neurobiological systems, including those underlying cognitive functioning. New parents must plan and be organized, but also prepared to respond to sudden change. That is, environmental cues need to be freshly evaluated, attended to, and efficiently processed so that the parent's behavior can be rapidly initiated or inhibited. New types of social interactions and physical features in the environment must be remembered to best guide future activities. Many such cognitive skills are enhanced in parental animals, and this involves a "resculpting" of their brain. This resculpting occurs to an impressive degree. For example, on a gross structural level, total brain volume shrinks by over 5% in pregnant women and this is accompanied by a 30% increase in the size of their ventricles.[664] These measures revert to prepregnancy values within 6 months after parturition, and at the same time the volume of women's gray matter in the prefrontal cortex and parietal lobe selectively increases.[665]

Research by Kinsley and Lambert was the first to discover that mothering enhances female rats' spatial memory, which is now known to involve striking hippocampal neuroplasticity. In an eight-arm radial maze, maternally experienced rats who had weaned their pups learned the location of baited arms after fewer trails than nulliparous females, and in a dry-land analog of the Morris Water Maze, the experienced females more rapidly reached a baited food well. The mothering experience, rather than pregnancy and lactation, is responsible for this benefit because nulliparous-sensitized females also show enhanced spatial memory.[666,667] Amazingly, these cognitive benefits of previous motherhood in rats can last for years.[668] Later work by Pawluski and colleagues extended this memory improvement in rats to current motherhood and found that it requires at least some contact with pups after parturition.[669,670] Similar behavioral results have been found in postpartum laboratory mice, and brain OT is one regulator of these effects in this species.[671] There is evidence that reproductive and maternal experience improves some memory capabilities in women,[672] and spatial memory improvements can also be found in rodent fathers.[673]

These results suggest that parental interactions with offspring, and in some cases exposure to hormones, induce plasticity in brain sites associated with learning and memory. Studies in rodents have focused on the hippocampus. One aspect of hippocampal plasticity in the maternal brain involves changes in the proliferation of new cells, including neurons. Cell birth in the brain was once thought to occur only during early development in mammals, but it is now well established in many adults, and altered cell proliferation is particularly common during times of hormone-mediated neurobehavioral flux, such as parenthood.[674] New brain cells are often visualized with the use of the cell birth date marker bromodeoxyuridine (BrdU), a thymidine analog readily incorporated into cells that are synthesizing DNA in preparation for mitosis.

One of the traditional areas for cell proliferation in the adult brain in many models is the granule cell layer of the hippocampal dentate gyrus (DG). Cell proliferation in the DG decreases during the middle and end of pregnancy in mice,[675,676] and during the first week postpartum in laboratory rats; the latter is a result of suckling-induced corticosterone release.[677–679] A similar decrease in DG cell proliferation is observed in parturient and maternal sheep.[680] The hormone dependence of this phenomenon is demonstrated by the finding that mothering experience alone in sensitized rats without the endocrine consequences of lactation *increases* cell proliferation in the DG (Figure 51.18).[678] A similar decrease in cell genesis during the first week postpartum also occurs in the midbrain dorsal raphe of laboratory rats,[643] which may be related to changes in serotonin output necessary for maternal behavior and lactation.

In forebrain sites more traditionally studied for roles in maternal behavior, increased numbers of BrdU-labeled cells are found in the dorsal BST and shell of the NA of postpartum rats that had brief maternal experience with pups compared to those without experience,[681] but females of various reproductive states do not differ in the number of BrdU-labeled cells in these sites or in their mPOA, ventral BST, or shell and core of the NA.[643] If new cells in these forebrain sites are not generated locally, they likely have migrated from the other classic neurogenic region, the subventricular zone (SVZ). The rodent SVZ shows pregnancy and early-postpartum changes in the number of BrdU-labeled cells, and this is a result of high circulating PRL.[682,683] In contrast, neurogenesis in the SVZ of sheep *decreases* after parturition and interaction with the lamb, which might somehow reflect differences between species in their need for

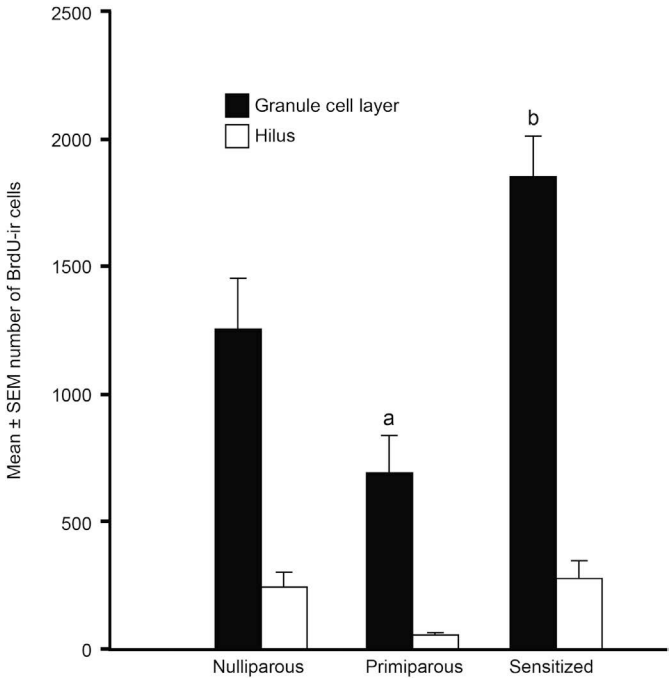


FIGURE 51.18 Number (Mean ± SEM) of BrdU-immunoreactive cells in the granule cell layer of the dentate gyrus of female rats measured 21 days after BrdU injection. Primiparous rats were injected with BrdU on postpartum day 1 and had fewer new cells surviving 21 days later compared to nulliparous control females. However, maternally sensitized nulliparous females had more new cells surviving 21 days after the beginning of pup exposure compared to controls. Groups did not differ in BrdU-immunoreactive cells in the hilus of the dentate gyrus. a = significantly different from nulliparous control females; b = significantly different from all other groups. *Source: Modified from Pawluski and Galea, 2007.*[678]

individual recognition of the offspring.[680] Many of these newly born cells in the SVZ of mice appear to migrate to the olfactory bulb, suggesting a role in the olfactory control of maternal behavior, but suppressing endogenous PRL during early pregnancy to block this neurogenesis impairs maternal behavior only when mice are tested under novel conditions, suggesting a nonsensory effect. Anxiety is increased in these PRL-suppressed dams, which may offer an explanation.[184]

The plasticity of the maternal hippocampus also involves structural changes in neuronal dendrites. CA1 pyramidal cells of late-pregnant and early-postpartum rats have a higher density of apical dendritic spines compared to that found in virgins, but, by the time of weaning, mothers have shorter CA1 and CA1 apical dendrite length and a lower number of branch points than virgins. This increase in apical dendrite spines depends on ovarian hormones,[684,685] and the later reductions in dendrite length and branch points may depend on adrenal corticosterone as it is not observed in multiparous females, which have lower circulating glucocorticoids compared to first-time mothers.[685] These results and the postpartum decrease in hippocampal cell proliferation discussed here might appear inconsistent with the improvement in short-term memory during lactation, but even regressive events in the brain could indicate an enhancement or refining of the neural circuitry involved in memory, and warn against a "more brain equals more behavior" type of logic. The functional significance of hippocampal plasticity may relate to maternal behavior toward pups. Lesioning the hippocampus before mating results in poor nest building, disorganized retrieval, and fewer pups surviving to weaning.[42] Similarly, mother rats with lesions of the fimbria, one of the major output paths of the hippocampus, build multiple small nests and retrieve some pups to each of them.[686] Unmated female rhesus monkeys that received hippocampal lesions during infancy, however, are normally affiliative toward unrelated infants,[687] which could reflect a species difference in the need for an intact hippocampus for mothering-like behavior or that other neural systems could eventually compensate for its loss in female monkeys. Peripartum hippocampal plasticity could also be involved in other functions in the females, including their altered negative feedback of the HPA axis response to stress.[167]

The cerebral cortex also exhibits peripartum plasticity, and cortical involvement in maternal behavior has recently received increased attention. Lactating rats have a thicker somatosensory cortex than cycling rats,[688] and twice as much somatosensory cortex is devoted to dams' representation of the skin of their ventral trunk, which is probably related to their enhanced sensitivity to pup probing and suckling.[689] This expansion of the somatosensory cortex requires physical contact with pups, and within 2 weeks after weaning its size reverts back to a prepartum state.[690] First-time mothers also have greater GFAP immunoreactivity in the cingulate cortex, a change that appears within hours of parturition if females are allowed to interact with pups, and this lasts through lactation.[691] Virgin female rats that are given exogenous hormones and interact with pups have a similar increase in cingulate GFAP immunoreactivity.[692] In addition, mother rats have a greater number of dendritic spines in the mPFC, an effect that coincides with their enhanced behavioral flexibility.[693]

Research on primates following lesions of the PFC or the anterior temporal cortex in postpartum rhesus monkeys demonstrated severe maternal behavior deficits, including an absence of contact seeking with infants, lack of retrieval from threatening situations, and only passive tolerance or active rejection if contact was infant initiated.[694,695] As discussed in the section Components of Parental Care, early work incorrectly suggested that no one area of the cortex was crucial for the expression of maternal behavior in rats but that the size of the cortical lesion was most relevant. We now know that specific regions of the PFC are activated by offspring cues and expression of parenting behaviors in rodents[628,696,697] and

that lesions of the medial PFC have profound effects on maternal behavior.[516,698,699] The PFC receives polymodal sensory input and has been implicated in stimulus recognition and executive functions contributing to attentional selection, optimal organization and planning, flexibility, and decision making in relation to complex goal-directed behaviors. The mPFC receives DA input from the VTA and has reciprocal connections with several subcortical structures, including many involved in maternal behavior such as the mPOA, BST, VTA, NA, and PAG. Thus, the mPFC can initiate a descending cascade though which the behavioral expression of a motivated or rewarding response results from outflow of the mPOA and limbic structures via the ventral pallidum and mesencephalic locomotor regions of the brain.[569]

The rodent mPFC can be functionally and anatomically subdivided into three distinct subregions—anterior cingulate (ACC), prelimbic (PrL), and infralimbic (IL)—each with distinct and dissociable contributions to the expression of maternal behavior across the postpartum period.[516] Permanent lesions and transient inactivation of the dorsal mPFC or ACC affect the organizational aspects of maternal behavior during both early- and late-postpartum periods, likely by inducing deficits in attention and behavioral inhibition processes, although mother rats remain very interested in pups.[41,43,49,516,698] Other studies have shown that depression of ventral mPFC activity severely disrupts maternal behaviors.[516,699] Specifically, transient inactivation of the infralimbic cortex, the most ventral part of the mPFC, severely disrupted all components of early postpartum maternal behavior, whereas inactivation of the prelimbic subregion did not.[516] It is worth noting that IL–mPFC inactivation, unlike mPOA inactivation that produced selective disruption of active maternal behaviors, resulted in females not directing their behavior toward pups at all but instead engaging in other activities such as eating, drinking, and resting. The IL–mPFC was also necessary for the dams' choice of pups over competing alternative conditioned incentives.[516] Together, it is tempting to speculate that the IL–mPFC is in a nodal position to integrate exteroceptive pup-related information with interoceptive information related to the maternal state, and to exert executive control to guarantee behavioral allocation toward pups and associated stimuli. By contrast, the mPOA is more a key component in motivational processing of pups and related incentive stimuli. Pereira and Morrell further demonstrated that, as the postpartum period progresses, the necessary facilitatory role of the IL–mPFC wanes, whereas the PrL–mPFC then contributed to the expression of late-postpartum maternal behavior.[516] This role transfer within subregions of the mPFC across lactation might be related to the transition from goal directed to habitual responding that probably occurs over time when dams continually interact with pups.

In summary of this subsection, the mPOA has a multifaceted role in promoting as well as inhibiting maternal behaviors. The mPOA is one of the most sensitive brain sites to ovarian and pituitary hormones and has reciprocal connections with neural sites processing all five sensory systems. It has dense connections with areas for the brain involved in motivated behaviors, emotion regulation, and cognition. The mPOA is perfectly situated to act as an integration site for the hormonal and sensory inputs required for the triggering or inhibiting of maternal interest, and then communicates that information with the motor, motivation, and cognitive systems necessary for the planning and performance of maternal behaviors (Figure 51.19).

Brain Control of Maternal Behaviors in Humans

Advances in fMRI to assess subregional changes in metabolic activity in the human brain have resulted in a small, but fascinating, collection of studies of how the postpartum human brain responds to infant-related auditory or visual cues. Because of the physical constraints involved with fMRI imaging, these studies do not reveal changes in brain activity during maternal behavior per se, but more about the perception and integration of sensory cues from infants and their rewarding or aversive properties. As discussed earlier in this chapter, heightened immediate-early gene expression in most brain sites involved in rodent mothering requires behavioral execution rather than distal stimulation from infants.[542,543,628] Evaluating the human brain response to infants in the absence of the ability to behave maternally

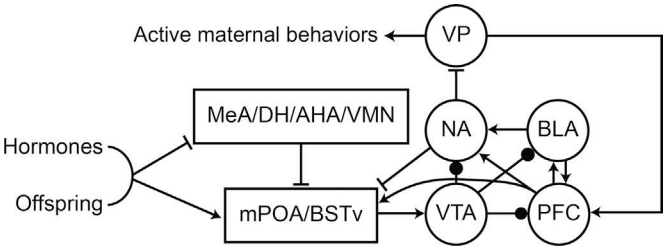


FIGURE 51.19 Hypothetical neural model for the stimulation of active maternal behaviors. The hormones of pregnancy and sensory cues of offspring suppress inhibitory input from the MeA–DH/AHA–VMN to the mPOA–BSTv while simultaneously stimulating mPOA–BSTv output to the VTA. This elicits dopamine release in the NA, PFC, and BLA. DA release in the NA inhibits VP output, thereby promoting the active components of maternal behavior. The NA, VP, PFC, and BLA can modulate this pathway by their connections to the MPOA–vBST or NA. AHA: anterior hypothalamic area; BLA: basolateral amygdala; BSTv: ventral bed nucleus of the stria terminalis; DH: dorsal hypothalamus; DA: dopamine; MeA: medial amygdala; mPOA: medial preoptic area; NA: nucleus accumbens; PFC: prefrontal cortex; VP: ventral pallidum; VTA: ventral tegmental area. Lines ending in arrows: excitatory input; lines ending in vertical bars: inhibitory input; lines ending in circles: DAergic signaling. *Source: Modified from Olazabal et al., 2013.*[750]

is nonetheless particularly appropriate because human parenting involves more higher-order cognitive and emotional integration compared to that required for the parental behavior of rodents and other subprimates.[229]

Experiments exposing mothers to the cries of their own infant or the cries of an unfamiliar infant reveal widespread cortical and subcortical activation. Reliability about what brain regions are activated by infant cries will increase as more studies emerge, but the sites most frequently reported across studies to be activated by infant cries are areas involved in sensory perception and processing (the thalamus and superior temporal and auditory cortex), those involved in reward (the striatum and nucleus accumbens), and numerous regions of the cortex involved in emotion and cognition (the insular, orbitofrontal–inferior frontal, medial frontal, temporoparietal, and fusiform cortices).[700] A smaller number of studies have reported activation in response to a familiar or unfamiliar infant cry in the anterior cingulate and ventral prefrontal cortices, amygdala, hypothalamus, hippocampus, and septum–preoptic area.[700] Other series of experiments using infant photographs or videos as stimuli have most often reported greater activation to one's own infant over an unfamiliar infant in many of the same areas activated by cries (e.g., the thalamus, striatum and nucleus accumbens, orbitofrontal and inferior frontal, and fusiform cortices). Activation in response to infant visual stimuli is also sometimes found in the insular and temporoparietal cortices, lentiform nucleus–globus pallidus, midbrain, and cerebellum. Thus, regardless of the modality of the distal infant cue, there is considerable overlap in the sites of the maternal brain that respond to these cues, and this may reflect the core neural network necessary for the sensory, emotional, reward, and cognitive processing involved in human mothering.

Metabolic deactivation is rarely found in the maternal brain during exposure to infant cues, but two groups have each reported deactivation of the medial frontal gyrus when subjects are exposed to the cry of their own infant when compared to the cry of an unfamiliar infant or when comparing the response to an infant cry and infant laughter.[700,701] Interestingly, lower activation of the medial frontal gyrus is also found when mothers view their own infant versus another infant.[702] The medial frontal gyrus is implicated in functions that include mood regulation,[703] self-referential processing,[704] and behavioral decision making.[705] In psychologically healthy and motivated mothers, this deactivation in response to cues from their own children could be associated with suppressing negative affect and the intention of providing to provide selfless caregiving to their infant.

Unfortunately, not all human mothers are healthy and motivated, and women are heterogeneous in their emotional and cognitive appraisals of infants. This is reflected by their fMRI activity. For example, mothers who are more sensitive have greater activity in their anterior prefrontal cortex, inferior and superior frontal cortex, and amygdala while hearing their own infant's cry compared to that seen in less sensitive mothers.[706,707] Studies examining how maternal mental health influences fMRI activity in response to infant cues demonstrate that poor maternal mood and high distress, which impair mother–infant interactions and bonding, are associated with lower amygdalar activity when mothers are viewing their infant's face.[708] Mothers with posttraumatic stress disorder, which is also associated with impaired bonding and interaction with infants, show limbic hyperactivity and lower prefrontal cortex activity than controls when watching videos of their children in a stressful situation.[709] Similarly, reduced prefrontal cortex activity is found in substance-using mothers exposed to infant pictures or cries.[710] Maternal brain activation in response to the cry of one's own infant compared to those from other infants is also lower in multiple cortical regions (superior and middle temporal, superior frontal, medial fusiform, and superior parietal) and in the caudate, thalamus, hypothalamus, and amygdala in women who deliver via Caesarian section rather than vaginally (Figure 51.20).[98] Interestingly, breastfeeding is associated with greater fMRI activity in some of these same regions (superior frontal cortex and amygdala) compared to the activation seen in mothers who bottle feed their infants,[707] and these differences have been proposed to reflect the neuroendocrine consequences of vaginal delivery and suckling, as well as differences in maternal sensitivity to the infant.[711]

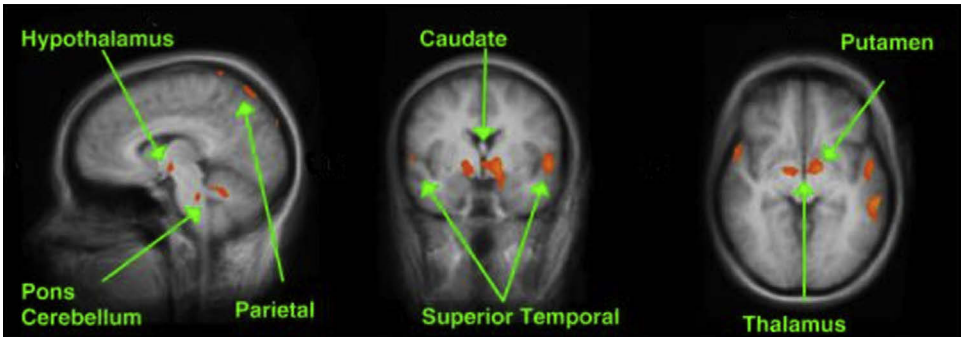


FIGURE 51.20 **Sites of the maternal brain where fMRI activity was higher (indicated by red) in mothers who delivered vaginally compared to via Caesarean section while listening to their own infant crying.** This figure is reproduced in color in the color plate section. *Source: Modified from Swain et al., 2008.*[98]

BRAIN CONTROL OF PATERNAL BEHAVIORS

Brain Control of Paternal Behaviors in Nonhuman Animals

Fathers in most biparental species show the same repertoire of caregiving behaviors as their female mates, including nursing-like postures, even though suckling by pups and milk letdown cannot occur.[326] Thus, it is parsimonious to think that brain networks controlling these behaviors are homologous to those in females, and immediate-early gene studies in prairie voles and California mice suggest this is mostly true. There has been no direct comparison of the immediate-early gene expression between female and male parents in either species, but patterns of immediate-early gene expression in parentally inexperienced virgin male and noninfanticidal virgin female prairie voles are very similar to those found in postpartum female rats and mice. For example, exposure to a single pup increases Fos expression in both the male and female prairie vole mPOA, BST, MeA, and lateral septum compared to the expression found after exposure to candy.[712] In California mouse fathers, Fos expression is elevated in the mPOA after exposure to just the distal cues from a pup.[713,714]

With regard to sex differences in Fos response, the magnitude of the increase in Fos expression in the prairie vole mPOA after exposure to a pup is much higher in virgin males than in virgin females, possibly reflecting the higher spontaneous parental responsiveness in males found in most studies.[330,715] Additionally, the paraventricular thalamus expresses Fos in paternally acting virgin male prairie voles, but it does not in females.[712] The relevance of this sex difference in prairie voles is unknown, but the paraventricular thalamus also expresses high Fos in postpartum mother rats,[542] and it could be related to sex differences in the role of the paraventricular thalamus in reward seeking[716] or HPA axis regulation.[717]

There have been relatively few studies manipulating the paternal rodent brain. Excitotoxic lesions of the virgin male prairie vole MeA and surrounding region slightly reduced sniffing and licking of pups, but greatly reduced huddling over and lying next to them. The effects were specific in that side-by-side contact with a familiar female was not affected by the lesions.[636] The role of the MeA in these males' behavior must be more complex than just processing olfactory stimuli from pups because olfactory bulbectomy produced much more dramatic deficits that included infanticide and disruption of other social and nonsocial behaviors.[718] In male California mice, electrolytic lesions of the mPOA produce results somewhat similar to those found in mPOA-lesioned female rats, including longer latencies to retrieve pups, less licking, and less time spent near offspring.[509,637]

The neurochemicals released from or acting in these sites in the paternal brain are surely numerous, but mostly unknown. In paternally experienced California mice, Fos is expressed in some serotonergic cells of the dorsal raphe.[714] A small number of the Fos-expressing cells in the BST and MeA of virgin male prairie voles contain tyrosine–hydroxylase and may release L-DOPA or dopamine to modulate their paternal behavior.[719] Fos is also expressed in the paternal prairie vole PVN, and quite a few of these cells synthesize OT or AVP.[336] In fact, numerous studies have focused on the role of AVP in paternal behavior. It has long been recognized that male prairie voles differ from nonpaternal species of voles in the density of V1a receptor binding in many areas of the forebrain, including having greater expression in their accessory olfactory bulb, cingulate cortex, BSTv, VP, central amygdala, and paraventricular thalamus but lower V1a binding in their lateral septum, lateral habenula, and PAG.[720] Correlational studies supporting a role for intracerebral AVP in fathering include that male prairie voles have plexuses of AVP-immunoreactive fibers in their lateral septum and lateral habenula (sites implicated in maternal behavior rats; see the section Components of Parental Care) that decrease in density after males cohabitate and mate with a female, and this decrease somewhat parallels the increase in their paternal responding. No changes were observed after mating in the AVP fiber plexuses of the nonpaternal male meadow vole.[328,721] Because mating increases AVP mRNA in the male prairie vole BST, which is the source of this fiber innervation, this drop in fiber density probably reflects increased AVP release.[327] Any causal link between this mating-induced change in AVP content and behavior is unclear because long-term castration of male prairie voles almost eliminates AVP synthesis in this system, but in some studies castration has no effect on their paternal behavior.[330]

Studies examining direct manipulations of central AVP on the paternal behavior of prairie voles have produced mixed results. Infusion of AVP into the border of the lateral and medial septum has been reported to increase males' time spent in contact with pups compared to saline-infused controls, while infusion of a V1a receptor antagonist reduced pup licking.[722] Some of these results could not later be reproduced,[723] and intracerebroventricular infusion of a V1a receptor antagonist has also been seen to have little effect on pup licking or other paternal behaviors,[724] but this latter null finding may be due to the nontargeted infusion. Even so, males receiving simultaneous intracerebroventricular infusion of a V1a receptor antagonist along with an OT receptor antagonist did display greater infanticide and reduced huddling over pups.[724] The effectiveness of the combined treatment may be related to the greater OT receptor expression in the frontal cortex, nucleus accumbens, BST, and paraventricular thalamus of prairie voles

compared to species of nonpaternal voles.[725] Lastly, intracerebral administration of AVP increases licking and huddling with pups and decreases pup-directed aggression in male meadow voles, which suggests that changes in central AVP are involved in switching on and off their facultative paternal behavior.[278]

Several correlations suggest that AVP is also associated with paternal behavior in male California mice. Within the hypothalamic PVN, there is a positive association between levels of AVP mRNA and males' approach toward a pup, but not other paternal behaviors.[726] Moreover, California mouse fathers have more AVP-ir cells and fibers in the PVN and supraoptic nucleus (SON) than do fathers of a nonpaternal *Peromyscus* species, *P. maniculatus*.[301] Within California mice, fathers that retrieve their pups more often have higher levels of AVP-ir in the SON.[727] There is also a transgenerational relationship between AVP and paternal behavior, with offspring raised by castrated fathers that display less huddling and grooming toward them later expressing higher AVP in the PVN.[728] These offspring raised by castrated fathers also display less huddling and grooming toward their own pups.[729] Together, these results from California mice suggest that AVP in the PVN is involved in adult and developmental plasticity of paternal behavior, perhaps related to the hypothalamic systems involved in stress.

Several lines of evidence also link AVP in the BST with developmental plasticity in paternal behavior. Cross-fostering litters between the more paternal California mouse and the less paternal and promiscuous white-footed mouse, *Peromyscus leucopus*, produce male offspring that are more similar to their foster parents with respect to pup retrieval.[730] AVP immunoreactivity in the offspring BST is positively correlated with the frequency of paternal retrievals that these cross-fostered male young had received and with their fathers' huddling, grooming, and time spent in the nest.[727] When retrieval by their fathers was increased by purposely displacing the litters from their nests, these offspring later retrieved their own pups more frequently (Becker and Marler, unpublished data) and also displayed higher paternal aggression after becoming fathers.[728] These male offspring that were retrieved more often by their own fathers also had more AVP immunoreactivity in the dorsal fiber tracts of the BST.[728] Moreover, there is a potential link between these results and testosterone because pups have an acute rise in testosterone when they are retrieved by their fathers,[731] and this could influence both their AVP system and future paternal behavior as adults. Paternal behavior in *P. californicus*, therefore, appears to mediate the behavioral transmission of both paternal retrieval behavior and aggression and may do so via plasticity of numerous central AVP pathways.

Central estrogen synthesis and presumably estrogen receptor signaling are required for paternal behavior in male California mice,[292] but estrogenic activity instead inhibits fathering in male prairie voles and possibly some other rodents. Correlational studies show that the paternal pine vole (*Microtus pinetorum*) has lower ERα expression in areas of the brain involved in paternal behaviors, including the MeA and BST, compared to the polygamous and nonparental montane vole (*M. montanus*).[732] Similarly, the highly parental male Djungarian hamster (*Phodopus sungorus*) has lower ERα expression in the BST compared to the less parental male Siberian hamster.[732] In prairie voles, regional populations of males that are more alloparental have lower ERα expression in numerous brain regions, including the mPOA, BST, and VMN, compared to populations of less alloparental males.[733] These differences in ERα expression in the MeA are functionally relevant for parenting because male prairie voles with viral vector-mediated overexpression of ERα in the MeA, but not in the BST, are much less likely to act paternally than controls.[734,735] This negative relationship between paternal behavior and ERα is specific to this steroid receptor because there are no associations between the brain distribution and/or density of androgen receptors[733] or progesterone receptors[736] and paternal behavior in adult male prairie voles.

Brain Control of Paternal Behaviors in Primates

The handful of studies of the paternal monkey brain have focused on neuropeptides, and the relationship between OT and maternal behavior in rodents appears to extend to male common marmoset fathers. OT levels released from hypothalamic explants of paternally experienced male common marmosets is significantly higher compared to those of inexperienced males,[737] and intracerebroventricular infusions of OT decreases the number of refusals that fathers made for food transfers to their offspring.[738] There are two studies associating central AVP with paternal behavior in common marmosets. One did not identify any changes in AVP release from hypothalamic explants based on paternal experience,[737] and the other found that fathers have a greater number of AVP V1a receptors in their prefrontal cortex, that they have a greater density of dendritic spines on pyramidal neurons in the prefrontal cortex, and that more of these spines contained V1a receptors compared to paternally inexperienced males.[739]

Because human fathers are more variable in their parental involvement and interact differently with infants than do mothers, the three published studies of fMRI activity in the paternal brain during infant cue exposure are an important contribution to the human parental brain literature (see the section Brain Control of Maternal Behaviors). Seifritz and colleagues found no specific sites activated in fathers compared to mothers listening to infant laughing or crying, but they did

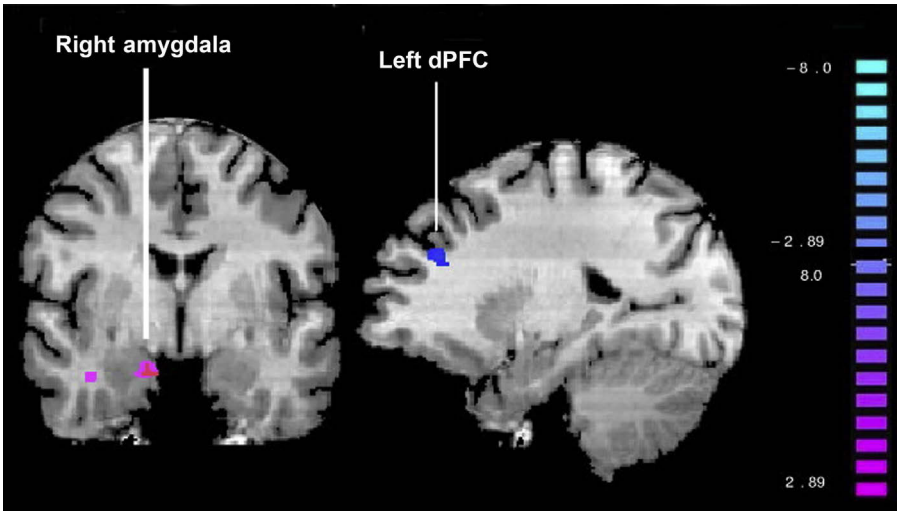


FIGURE 51.21 Sites in the parental brain where mothers (left) or fathers (right) showed greater fMRI activity in response to their own infant video compared to viewing an unfamiliar infant. This figure is reproduced in color in the color plate section. Mothers showed greater activity in the right amygdala and temporal, occipital, and parietal cortices compared to fathers, while fathers had greater activity in the dorsal prefrontal cortex (dPFC). *Source: Modified from Atzil et al., 2012.*[711]

discover a few overall sex differences in activity, with women (mothers or nonmothers) showing deactivation of the anterior cingulate cortex and mesial prefrontal cortex during cue exposure, whereas men (fathers or nonfathers) showed no such change.[701] The authors attributed the findings to sex differences in how these cortical areas control sensory gating and worrying or anxiety. They also found that compared to nonparents, parents of both sexes exhibited greater activation to infant crying in the amygdala; the cingulate, ventral prefrontal, and insular cortices; and the temporoparietal junction—all perhaps related to socioemotional regulation. A recent study comparing fMRI activity while fathers viewed video clips of a doll or their own infant's emotionally neutral face found greater activation to the infant in a host of cortical regions that are also often activated in mothers (orbitofrontal, lateral frontal, superior parietal, and midtemporal). Some cortical regions (orbitofrontal, inferior and superior frontal, supramarginal, and midtemporal) were also more metabolically active when fathers viewed their own versus an unfamiliar infant.[740] By imaging both mothers and fathers watching the same videos of their infant playing alone or a previous interaction with them, Atzil et al. found that functional activity was correlated between father-and-mother pairs in numerous cortical areas involved in empathy and social information processing (anterior cingulate, inferior frontal, inferior parietal, medial and lateral prefrontal, and insula).[711] Unlike the findings of Seifritz et al. indicating no father–mother differences in fMRI activity in response to infant vocalizations,[701] Atzil and colleagues further found that mothers showed higher activation to their own infants' visual cues than did fathers in areas of the cortex (temporal, postcentral, and fusiform), caudate, and amygdala (Figure 51.21).[711] Also only in mothers, activity of the amygdala, cingulate cortex, and nucleus accumbens was positively correlated with their plasma OT levels. Paternal plasma OT was instead negatively correlated with activation in cognitive cortical regions (dorsolateral PFC, dorsal ACC, IPC, and precentral gyrus),[711] and these results could possibly be related to sex differences in limbic and cognitive control of parenting.

CONCLUSION

A substantial amount of information clearly exists regarding the endocrine, neuroendocrine, sensory, and neural substrates necessary for the onset and maintenance of parental behaviors in rodents, sheep, and primates. We believe there are still numerous broad and specific areas for future research, and a few points to consider about the existing literature, that would improve the understanding of the physiology of parental care. Specifically, these might include:

- Increased investigation of the involvement of some steroid hormones in mothering. There is a very notable gap in our comprehension of how and where in the brain progesterone inhibits or facilitates caregiving behaviors. Since the early studies by Numan,[578] in which progesterone implanted into none of the hypothalamic or midbrain sites examined could delay the onset of mothering, there has been no direct investigation of this question. Studies using targeted implants of progesterone into brain sites not examined in that study, or brain site–specific knockout of PR, would be informative. There is also almost nothing known about any behavioral implications of the very high androgen secretion during pregnancy or where in the brain androgen receptor activity might influence the onset of mothering.

- A more informed perspective is needed on the redundancy existing among the endocrine factors underlying parenting. As discussed in this chapter, knockout models in which parental behaviors are unaffected or less impaired than expected suggest that such redundancy exists and provide insight into the systems most valuable for further analysis of this question.
- The neurobiological mechanisms involved in how parental behaviors are maintained by pup sensory cues after the endocrine effects have waned have not been well studied. Are the cells that were affected by hormones the same cells that are later responding to pup sensory cues? How is the physiology of these cells altered by hormones to assume this function? A somewhat similar question arises regarding how the multiparous brain differs mechanistically from the primiparous brain. The neural systems involved in mothering have been permanently altered to require reduced or no hormone exposure and less sensory input to elicit maternal behavior, but additional research into how this occurs would be tremendously valuable.
- We and many others have reviewed the scientific literature on the numerous neural systems involved in the suite of behavioral alterations that are necessary for successful parenting, but how these systems interact as a larger neural network for seamlessly integrated behavioral responding is not well understood. Furthermore, because these systems are involved in almost every social behavior,[6,741] it would be important to know how they selectively respond to offspring. Are different populations of cells within these structures involved in particular behaviors? How do the unique endocrine events of pregnancy versus other endocrine events involving the exact same ovarian and pituitary hormones (e.g., the estrous cycle) affect cells in ways that determine what social cues are responded to and what cues are ignored?
- As translating information from nonhuman animals to humans continues to become an important goal of the field studying maternal behavior, animal models must be chosen wisely. For example, while studying nonhuman female primates is admirable, it is obviously difficult or impossible for most researchers. A cogent argument has been made that one of the animal models closest to humans in their peripartum endocrinology, delivery of a small number of precocious offspring, mother–offspring communication, and social attachment is the now rarely studied guinea pig.[742–744]
- Carefully consider the existing and future literature in the context of the natural display of parenting behavior. Beginning with some of the foundational work in this field on laboratory rats[15,32,37] there has sometimes been an emphasis on retrieval of offspring as the most relevant, or sometimes the only, outcome measure. Retrieval is convenient to assess because it is usually rapid and clearly displayed by a subject or not, but it is problematic for a more holistic understanding of parenting because retrieval is uncorrelated with other parental behaviors of the same animals after retrieval has concluded or when the subjects are observed under undisturbed conditions.[745,746] It is also problematic because this type of long-distance carrying of young is probably rare in nature unless the nest site is disturbed.[747] Importantly, because parenting is not a unitary process, but instead involves a collection of individual behaviors with different sensory, endocrine, and neurobiological determinants, factors influencing retrieval will not translate to all other components of offspring care.
- With few exceptions,[661,678,748] we as a field focus on first-time parents with no previous experience with young. As discussed in part in this chapter, this is unnatural because most adult mammals are unlikely to approach parturition with no previous parenting experience because they would have received it during juvenile alloparenting and/or had given birth multiple times before. Under natural conditions, therefore, the relevant endocrine and sensory cues most often act upon an already heightened baseline to promote maternal behavior. Thus, laboratory studies may reveal how powerfully hormones, pup sensory cues, and other factors can influence parenting, but their importance for the majority of mothers and fathers living in natural environments is probably overemphasized in a literature that is mostly based on laboratory studies of inexperienced animals.

Parental behaviors directed toward offspring can tremendously help or materially harm the young, and can do so with lifelong consequences. As researchers who benefit from and struggle with the advantages and limitations of our models, we must not lose sight of the idea that our knowledge obtained from nonhuman models of parental behavior is both incredibly useful in its own right for a comparative perspective about social–behavioral endocrinology, and also informs the human condition in ways that can immensely benefit both parents and offspring during this critical phase of mammalian reproduction.

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CHAPTER

52

Epigenetics of Reproduction

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INTRODUCTION

Historical Overview

The idea that gene expression is modified by environmental and experiential factors is not new. Waddington first articulated the developmental dilemma that all cells have the same DNA and yet they are differentiated to perform vastly different functions.[1] Psychologists such as Irving Gottesman observed that identical twins were hardly clones, and their behavior and appearance was even more divergent when reared apart.[2] Thus, the fact that genes are not the only source of phenotypic variability has been well accepted for many years. What is new is that advances in molecular biology have provided us with the tools to discover mechanism that mediate changes "above the genes".

Figure 52.1 illustrates the exponential growth in publications on epigenetics since the first papers were published in 1958.[3,4] Work on reproduction in the context of epigenetics is also accelerating and has grown markedly in the early twenty-first century. Recognition of hypermethylation as the major cause for X chromosome inactivation pushed the area ahead.[5] Silencing of the X chromosome is now one of the best examples of epigenetic regulation of gene expression. In the early 1990s, David Barker and colleagues discovered that infant birth weights were correlated with risk for a variety of adult-onset diseases, including diabetes and hypertension[6–8] (see also Chapter 45). This hypothesis, now interpreted broadly as developmental origins for adult disease, has been extremely influential and has been a major intellectual breakthrough in reproduction as applied to environmental antecedents of well-being. Recognition of the important role that maternal care has in behavioral phenotypes is also a breakthrough—and with it, the application of epigenetics to neuroscience.[9] In neurons, changes in cellular morphology (synapses, spines, etc.) can influence cell function in a highly plastic manner, and environmental variables are known to cause these changes. Thus, in the brain, the important distinction between epigenetic modifications that may only last for a generation vs those that are permanent and inherited is particularly relevant to studies of reproductive physiology and behavior. The short-term epigenetic modifications can be thought of as "socially transmitted" as compared with germline transmission of long-lasting changes. The coming years will prove to be an exciting time for epigenetics and reproduction. We now know that nearly all epigenetic enzymatic reactions are reversible. New levels of epigenetic modification are being revealed; as they emerge and are tested, most have applications in reproduction. Not only is epigenetics providing us with solutions to long-standing problems in reproduction, it also has caused us to rethink some long standing dogmatic notions.

Nowhere is the importance of epigenetic regulation of gene expression more self-evident than in reproduction. Only through restrictive modifications of the genome that tightly control the expression of one suite of genes over another can a single fertilized egg give rise to the rich diversity of cell and tissue types found in mature organisms. This fantastic panoply of development is orchestrated both temporally and spatially by epigenetic

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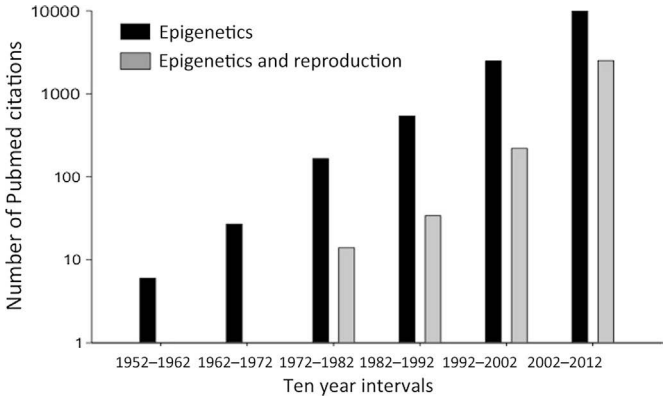


FIGURE 52.1 Publishing trends for epigenetics. A survey of the digital scientific data base PubMed Central from the National Library of Medicine USA (http://www.ncbi.nlm.nih.gov/pmc) reveals a continuous rise in the number of citations in which the term *epigenetics* is a key word and a parallel increase in the number that include both epigenetics and reproduction.

regulation of gene expression. Once complete, this process further serves to imprint upon the new organism features of its environment and experience and, if salient enough, to ensure that the impact of these features are passed on to the next generation in anticipation of similar circumstances. The centrality of epigenetics to reproduction is further evident in fundamentals such as X inactivation, parental imprinting, and the organizational actions of steroids on almost all tissues and organs, including the brain. The goal of this chapter is to review the basic mechanics of epigenetics and then to place them in the context of reproduction. We will review the two best described processes: histone modifications and DNA methylation. We will also describe some of the techniques now in use in the field and we will provide a survey of epigenetic mechanisms in selected topics.

Reproduction is the main mechanism that implements evolution. Evolution acts by natural and sexual selection, favoring individuals that are able to adjust to the ambient environment and then survive for long enough to reproduce successfully and pass genetic material on to subsequent generations. When the environment is stable and energetic demands are not severe, reproduction may be a rigidly encoded process. For example, the yeast *Saccharomyces cerevisiae* has two mating types that participate in different forms of reproduction: asexual budding and sexual reproduction. In predictable environments, mitosis is the predominate form of population growth. However, in complex environments, sexual reproduction, meiosis, and gamete formation are favored. In multicellular organisms that have obligate sexual reproduction, the capacity to respond to environmental pressures and to change breeding strategies can occur via maintenance of some genes accompanied by silencing of others, via long-term adaptation. However, when environments change in real time, other processes and rapid accommodation are required. Epigenetics is undoubtedly one way that organisms respond to changes in the environment and accomplish rapid adaptation.

The examination of epigenetics in reproduction is relatively recent. Investigation of epigenetic regulation immediately postfertilization is the most mature science with substantial advances and landmark achievements, some of which will be discussed below. Newer to the field is the investigation into epigenetic regulation in the adult and developing brain; here, we give special attention to aspects of this relevant to the physiology of reproduction.

Definitions of Epigenetics

The term *epigenetics* is derived from the Greek use of the prefix *epi* with a root word to indicate relatedness that involves being above, on, over, after, and so on. In this context, it is taken as anything that involves the genome but does not result in changes to the nucleotide sequence of the DNA. Examples of what is considered "epigenetic" can vary from being quite restrictive to fairly broad. Moreover, the definition and function of epigenetic modifications is rapidly changing as research progresses. For purposes of this discussion, we will start with one major distinction—the epigenetic modifications required for permanent differentiation of cells and tissues and which generally follow a program vs the modifications that are responsive to the immediate environment and experience. We define environment and experience in the broadest terms, such as the internal steroid milieu, maternal diet, ambient temperature, psychosocial stress, or exercise. The latter type requires an "epigenerator", a variable that induces the initiation of an epigenetic modification, followed by maintenance of that modification.[10] Regardless of the nature of the epigenerator, epigenetic changes can be further divided into those that will endure into multiple subsequent generations via the germ line, which are therefore called *germline-dependent* epigenetic changes, vs those that will endure for some shorter, variable length of time. These changes may only occur during a portion of the organism's lifetime, or they may even impact the next generation but not beyond; these have been referred to as *context-dependent* epigenetic changes[11] (Figure 52.2).

The two mainstays of epigenetic change are those directed to histones, which modify the structure of chromatin, which surrounds the DNA, or to more specific methylation changes at selected regions of the DNA. Modifications to histones are far more complex because they most commonly involve methylation and/or acetylation, but they can also occur via a number of other processes and combinations thereof. We discuss both forms in considerable detail, including that in either case the ultimate effects of the epigenetic change are consistently

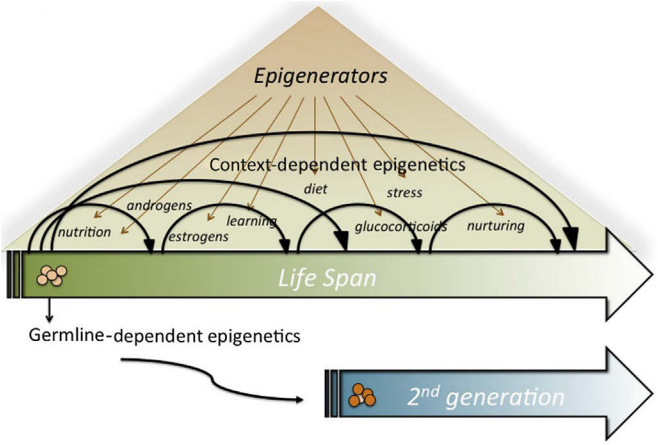


FIGURE 52.2 Epigenetic overview. Epigenetic changes are generated (the epigenerators) by extrinsic or intrinsic variables that activate the enzymes mediating methylation of nucleoside residues in the DNA and modifications to the histones in the surrounding chromatin. Typical epigenerators include gonadal steroids, stress hormones, nutrition, and experiences such as those associated with maternal care or learning. Epigenetic changes can be context dependent and last only a single lifetime, or they can be incorporated by the germ line and therefore be transgenerational. Heritable epigenetic changes are those that endure beyond the F2 generation.

manifested by suppression and/or enhancement of gene expression.

A broader inclusion of mechanisms that produce epigenetic changes includes consideration of the following: so-called microRNAs (miRNAs), which are noncoding RNAs (ncRNAs) such as those involved in inactivation of one X chromosome in every cell in females; parental imprinting, resulting in preferential monoallelic expression; and the appearance of retrotransposons in the DNA, previously thought of as junk but now known to be both a source of and contributor to genetic variation (see below). In addition, it is important to remember that new mechanisms are being and will be discovered; thus, this list is not exhaustive.

Chromatin Remodeling

Chromatin consists of DNA and specialized protein structures that comprise the nucleosome. These specialized protein structures are the histones. Chromatin has multiple functions, including the critical structural aspects of efficient packaging of the DNA and provision of the tensile strength needed for mitosis. In the context of epigenetics, the most important function of chromatin is to regulate gene expression by controlling the access of transcription factors and associated proteins to the DNA. When chromatin is tightly compacted, access to DNA is blocked and transcription is reduced. In contrast, when the chromatin is loosely associated with the DNA, conditions are permissive for transcription because the nucleotide sequence is accessible to the ensemble of transcription factors.

Chromatin comes in two basic varieties, heterochromatin and euchromatin, distinguished initially based on the cytology density of staining, with heterochromatin staining more darkly due to its dense packaging. Heterochromatin represents the vast majority of chromatin and it is not uniform, with different domains distinguished by the degree and nature of epigenetic modifications. The heterochromatin making up the centromeres and telomeres is the most densely packed and immutable, and it is referred to as constitutive heterochromatin, while other regions of heterochromatin can be modified and thereby change the profile of gene expression nearby or in neighboring regions. Euchromatin is the site of active gene expression, although not all genes within a euchromatin segment are capable of being activated. Both types of chromatin are of interest in the epigenetics of reproduction.

Histones and Nucleosomes

The human genome contains approximately 25,000 genes, but their sequence makes up just a small fraction of the over 3 billion base pairs contained in the chromosomes. All this genomic material needs to be compacted to fit inside the cell nucleus; yet, the relevant DNA sequences have to be accessible for transcription. The solution to this dilemma is that condensed DNA is wound around a set of core histone proteins: H2A, H2B, H3 and H4. Approximately 145–147 base pairs of genomic DNA wrap around a set of eight histones, which is considered the basic unit of chromatin, and is referred to as the *nucleosome*. Among the four core histone proteins, the H3 and H4 partner as do the two H2s. Within the nucleosome, each histone is found in duplicate. A fifth histone, H1, serves to link the cores to each other. Thus, the histones are built as octomers and are among the most highly conserved proteins across all species. This condensation and basic architecture is illustrated in Figure 52.3. While the classic "beads on a string" structure is helpful for conceptualizing how DNA is condensed, the more we learn about chromatin structure, the more variations we uncover. Histone variants are relatively common, particularly for H2A and H3. The histone variants are species, and in some cases tissue, specific[12] and mutants have been found associated with disease. Clearly, this is an area poised for further discovery.

Histone Modifications and the "Histone Code"

Not only do the histone proteins serve as a framework for condensation of the chromosomal material, they also facilitate and/or block accessibility of DNA to transcription. About 25% of the mass of the histones is composed of N-terminus, or tail, domains.[13] When the

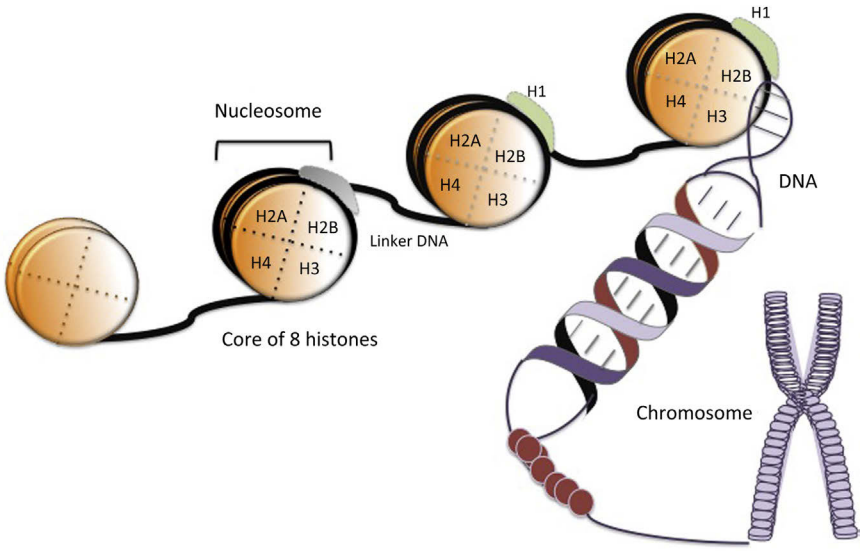


FIGURE 52.3 Histones and nucleosomes. Chromosomes are comprised of DNA and associated proteins, collectively known as chromatin. Condensed DNA is wound around a set of core histone proteins: H2A, H2B, H3, and H4. Approximately 145–147 base pairs of genomic DNA wrap around a set of eight histones, which is considered the basic unit of chromatin and is referred to as the nucleosome. Among the four core histone proteins, the H3 and H4 partner as do the two H2s. Within the nucleosome, each histone is found in duplicate. A fifth histone, H1, serves to link the cores.

tails are exteriorized, they are accessible to a number of enzymes. The histone tails twist inside and outside of the chromatin. These flexible N-terminal tails are fluid in their conformation and can serve to either increase or reduce the density of the chromatin structure. Importantly, nucleosome structure is not uniform. Other structures for the nucleosome have been solved, and these reflect variations in DNA compaction and interactions with histone and nonhistone proteins. At least 60 different residues on histones are known, and this is certainly an underrepresentation. Moreover, many types of histone tail modifications have been identified, and it is likely that this is not exhaustive. The possible histone modifications now include nearly all the known protein modifications: acetylation, methylation, proline isomerization, sumoylation, ubiquitination, phosphorylation, ADP ribosylation, and deamination. These modifications occur primarily on lysine residues,[14] but they can also occur on arginine, tryptophan, and serine residues. In addition to changing access to DNA, histone tails also modify the relationships between the nucleosomes. Seemingly small changes in the primary structure of chromatin can be amplified to affect the shape, stability, and/or protein surfaces of these complexes.

The "histone code" was proposed by Strahl and Allis to take into account the possibility that combinations of posttranslational modifications (PTMs) may interact. Like the genetic code, where certain stretches of amino acids or nucleotides have specific functions, such as stop or start codons, perhaps combinations of enzymatic modifications on H3 lysines formulate specific transcriptional regulatory directions. Moreover, the combined actions of certain modifications may be different from those produced in isolation.[13] The numbers of possible combinations of modifications and their timing all contribute to the complex manner in which DNA transcription is regulated. The histone code postulates that combinations of modifications will have meaningful readouts similar to DNA base pairs. The code may explain why a certain type of modification, such as acetylation, typically enhances transcription the majority of the time; however, under some conditions, when other modifications are present, it may be repressive (Figure 52.4).

Position is also influential. Two PTMs that are in *cis* (on the same histone) may have a different outcome on transcription than the same two PTMs in *trans*, which can occur on the same nucleosome or adjacent nucleosomes. One example of this is on histone 3 (H3). If H3 serine 10 (S10) is phosphorylated, the HP1 chromodomain, an enzyme that "reads" PTMs, cannot bind to the H3 lysine 9 trimethylation (K9me3) site.[15] Another example is the case of the bromodomain, an extraterminal domain family of nuclear proteins. These proteins act as transcription factors and synchronize the activities of two acetylation sites on histone H4.[16,17]

Histone-Modifying Enzymes

One way to consider the enzymes associated with chromatin modification are that they transduce the signals initiated by the epigenerators. Thus, if the organism experiences a change in ambient temperature, social conditions, or dietary conditions, for example, these are interpreted or "read" by epigenetic initiators, such as ncRNAs and DNA binding factors. Then, the new epigenetic status is maintained by histone-modulating enzymes,[18] which are often referred to as "writers" and "erasers". The dynamic aspects of the changing chromatin structure are regulated by modifications on specific amino acids in the N-terminal tails. These amino acids are the sites for enzymatic action. The two best-understood modifications are acetylation and methylation. Acetylation only occurs

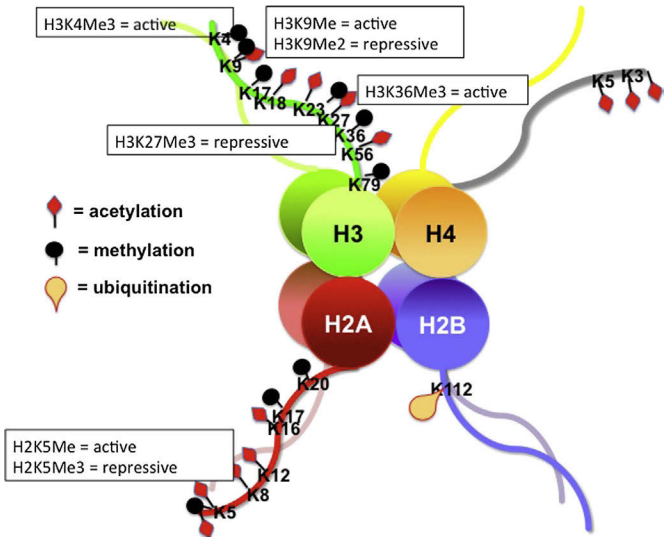


FIGURE 52.4 The histone code. The term "histone code" was proposed by Strahl and Allis and refers to the impact that combinations of modifications to the histones has on gene expression, either repressive or permissive. The type of modification (i.e., acetylation, methylation, phosphorylation etc.), the histone on which it occurs (i.e., H3 vs H4), and its position on the histone tail all impact the final readout regarding transcription. Many modifications occur on lysines (K), but serines are modified as well. Methylation of lysines can be transcriptionally active or repressive depending on the number of methyl groups added. Acetylation of lysines is predominantly activating but the nature of its effects can be changed by alterations to other residues either *cis* or *trans*.

on lysine residues. When positive charge is removed, the interaction of the histone tail with the negatively charged phosphates on DNA is reduced. Thus, euchromatin can be transcribed. The enzymes that regulate this process are histone acetyltransferases (HATs), also known as "writers", which facilitate acetylation. Epigenetic mark removal is conducted by "erasers". The histone deacetyltransferases (HDACs) remove acetyl groups from lysine residues. Specific lysine residues and the HATs that interact with them are shown in Table 52.1. Importantly, HATs and HDACs act as part of multiprotein complexes that recruit other enzymes and proteins to specific sites. This is important to keep in mind because simply measuring HDACs alone is not enough to understand activity levels. If the appropriate DNA complexes are not present, the HATs or HDACs are not recruited to the target sites and will not affect gene expression.

HISTONE ACETYLTRANSFERASES

There are several classes of HATs, based on their functions and their cellular distributions. Type A HATs are nuclear and type B HATs are present in the cytoplasm. We will limit our discussion to type A HATs, which are further refined into five subtypes.[74] One class of type A HATs is related to nuclear receptors and includes steroid receptor coactivators 1 and 3 (SRCs). CREB-binding protein (CBP) is in its own grouping, which is often denoted as CBP/p300 because these two proteins are structurally very similar and may substitute for each other (see Chapter 9). However, separate functions have been discovered, including modifications of different lysine residues.[75] Another type A HAT group is the GNAT family. The first HAT discovered was Gcn5, which can coactivate the estrogen receptor alpha (ERα; p300/CBP-associated factor). The MYST family of enzymes is important for differentiation and development. One example of cross-talk between these groups of HATs is the ability of ERα-CBP/p300 to stimulate histone acetylation.[76] Acetylation has actions in addition to changing the charge of N-terminal tails on histones; for example, acetylated histones serve as docking sites for other proteins that recruit transcription factors. HATs also function in DNA damage detection and repair.

HISTONE DEACETYLTRANSFERASES

Epigenetic mechanisms can be silenced during various stages in development and cellular differentiation. Presently, there are 18 known deacetylating (HDAC) enzymes, which in general act to suppress gene transcription by removing acetyl groups from lysines. These enzymes act in complexes that often include histone methylating or demethylating enzymes. In addition, the HDACs can act on other substrates. HDACs are grouped into five classes based on their sequence similarities to three yeast HDACs. All the classes are somewhat homologous, with the exception of Class III containing the sirtuins, which are dependent on nictotinamide adenine dinucleotide (NAD) and are important for cellular metabolism.

Class I includes HDAC1, 2, 3, and 8, which are found in cells throughout the body in several multiprotein complexes. These nuclear complexes are highly conserved and can associate with two families of histone demethylases. HDAC1 and 2 are part of complexes that generally function in development. In the brain, HDAC1 is mainly present in glia and HDAC2 is expressed primarily in neurons. When either one of these HDACs is knocked out in mouse brain, the mice survive. But when both are deleted, the mice do not survive past a week after birth and brain development is severely abnormal.[77] Both HDAC1 and 2 participate in the Notch cell signaling pathway during embryogenesis.[78] One set of HDAC complexes act primarily in promoter regions and to a lesser extent at transcribed regions. Another HDAC complex, NCoR/SMRT, also including HDAC1 and 2 along with methyl binding domain proteins, is essential for development in many species from flies to mammals. NCoR/SMRT binds with nuclear receptors and together they recruit other proteins to DNA. In conjunction with the repressive element silencing transcription (REST) complex, NCoR is involved

TABLE 52.1 Histone Modifications with Associated Enzymes

Acetylation					
Histone	**Modification Site**	**Histone Acetyltransferases**	**Histone Deacetyltransferases**	**Proposed Function**	**References**
H2A	K5	Tip60, p300/CBP		Transcription activation	19,20
H2B	K5	p300/CBP, ATF2		Transcription activation	20,21
	K6		HDAC9	Transcription activation	
	K12	p300/CBP, ATF2		Transcription activation	20,21
	K15	p300/CBP, ATF2		Transcription activation	20,21
	K20	p300/CBP		Transcription activation	20
H3	K9	Gcn5, PCAF, unknown	SIRT1	Histone deposition transcription activation	22–24
	K14	GCN5, PCAF, Tip60, ELP3, p300/CBP Hpa2, Sas2, Sas3, Rsc4	SIRT1	Histone deposition, transcription activation and elongation, DNA repair, RNA polymerase III transcription, RNA polymerase II transcription, euchromatin	22,24–29
	K18	GCN5, PCAF, p300/CBP	SIRT7	Transcription activation, DNA repair DNA replication	20,23,30,31
	K23	GCN5, Sas3 p300/CBP		Histone deposition, DNA repair transcription activation (elongation)	22,23,30
	K27	GCN5		Transcription activation	32
	K56	Rtt109	Hst3/4	Histone deposition, chromatin assembly, DNA repair	33
H4	K2	Sas2, Tip60, Hbo1	SIRT2		
	K5	Hat1, Tip60, ATF2, Hpa2, p300/CBP, Hbo1		Histone deposition, transcription activation, DNA repair, unknown	20,21,26,27
	K8	GCN5, PCAF, Tip60/PLIP, Hbo1, ATF2, ELP3, p300/CBP, Bdf1		DNA repair, transcription activation (elongation)	20,26–28
	K12	Hat1, ATAC, Tip60/PLIP, Hpa2, p300/CBP		Histone deposition, telomeric silencing, transcription activation, DNA repair unknown	20,26,27,34
	K16	GCN5, Sas2, Mof, Tip60/PLIP, ATF2, Sas2, TAF1	SIRT2-3	Transcription activation, DNA repair, euchromatin	26,27
	K79	Hat4		Nucleosome assembly, DNA repair	35
	K91	Hat4		Nucleosome assembly, DNA repair	35

Methylation					
Histone	**Modification Site**	**Methyltransferases**	**Demethyltransferases**	**Proposed Functions**	**References**
H1	K26	EZH2	SIRT1	Transcriptional silencing	36,37

TABLE 52.1 Histone Modifications with Associated Enzymes—cont'd

Histone	Modification Site	Methyltransferases	Demethyltransferases	Proposed Functions	References
Methylation					
H3	R2	CARM1, PRMT5,6,7	JMJD6	Transcriptional repression, transcriptional activation	38
	K4	Set 7/9, MLL1-5, Set1A/B, ASH1, Chd1, BPTF, ING2, PRDM9, SMYD3, LSD1	LSD1, LSD2, SMYD3, NO66, JMJD2A, FBXL10/11, KDM5A, 5B, 5C, 5D, 7B, NO66	Transcriptional activation	39–41
	R8	PRMT5		Transcriptional repression	42
	K9	SUV39H1/2, Clr4, G9a, EHMT1, ADD, ANKYRIN, EZH2, Hp1Eset/SetdB1, Cll8, Riz1, UHRF1	LSD1, KIAA1718, PHF2, PFH8, JMJD2D, JMJD1A, JMJD2A, 2B, 2C, 2D, JMJD3A, PRDM2, FBXL10/11, JMJD3, 2C, 5qNCA	Transcriptional silencing, transcriptional repression, genomic imprinting	43–46
	R17	CARM1		Transcriptional activation	30
	R26	CARM1		Transcriptional activation	38
	K20	PR-Set7/8, SUV4-20H1/2, Kmt3A, 3B, 3C			47
	K27	EZH1/2, EZH2, G9a, PC	EZH1, KIAA1718, KDM6A, 6B, 6C, 7B, SIRT1	X-inactivation transcriptional silencing	45,48
	K36	SETD2, Nsd1/2, NSD3, NO66,	JMJD2A, 2B, 2C, 2D, 3 JMJD5, FBXL10/11, NO66, Kdm7B	Transcriptional activation (elongation)	49
	K79	DOT1L		Euchromatin, transcriptional activation (elongation), checkpoint response	50–52
H4	R3	PRMT1, PRMT5-7	JMJD6	Transcriptional activation, transcriptional repression	13,42
	K16	Mof	SIRT1		53
	K20	PR-Set7/8, Suv420H1-2, BAH, Chromo-barrel, L3Mbtl1, EZH2	SETD8, PHF8	Transcriptional silencing, heterochromatin	54,55
	K59	Unknown		Transcriptional silencing	56

Histone	Modification Site	Enzyme	Proposed Function	References
Phosphorylation				
H1	S27	Unknown	Transcriptional activation, chromatin decondensation	36,37,57
H2A	S1	MSK1, unknown	Mitosis, chromatin assembly, transcriptional repression	58
H2A.x (variant)	S139	ATR, ATM, DNA-PK	DNA repair	59–61
H2B	S14	Mst1 unknown	Apoptosis DNA repair	62,63
H3	T3	HASPIN/Gsg2	Mitosis	64
	S10	AURORA-B kinase IKK-α Snf1, 14-3-3 protein	Mitosis, meiosis transcriptional activation	65–68
	T11	DLK/Zip	Mitosis	69
	S28	AURORA-B kinase MSK1, MSK2	Mitosis immediate-early activation	70–72
H4	S1	CK2, unknown	Mitosis, chromatin assembly, DNA repair	57,73

New histone-modifying enzymes are still being discovered and this list is not comprehensive. In addition, nomenclature for these genes may have changed.
L = lysine, R = arginine, S = serine, T = threonine. Enzymes designated in lower case were identified in yeast.

in neural and glial development.[79–81] Not only do these protein complexes have independent actions, but they also work together in many cell types.

Class IIa HDACs include HDAC4, 5, 7, and 9. Unlike the Class I HDACs, these enzymes are more restricted in their distribution, yet they can exist in the cytoplasm as well as the cell nucleus. Class IIa HDAC enzymes are abundant in heart, skeletal muscle, and brain (particularly HDAC4 and 5), and HDAC7 is expressed in a subset of thymocytes. HDAC6 is found in testes and HDAC10 is present in liver, spleen, and kidney.[82] Several lines of evidence suggest roles for Class I HDACs along with HDACs 4 and 5 in learning and memory.[83,84] These two HDACs are highly expressed in the basal ganglia, hippocampus, and cerebellum. Class IV contains only HDAC11, which is widely distributed in the developing brain; after birth, expression increases in specific regions.[85] In particular, HDAC11 is concentrated in adult oligodendrocytes and hippocampal granule neurons.

Deciphering the roles of these important enzymes in the brain is in its early phase. A descriptive study examined co-expression of HDACs and various neuropeptides, including corticotropin-releasing hormone (CRH), oxytocin, vasopressin (AVP), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), orexin, histamine, dopamine, serotonin, and noradrenaline neurons. Unique co-expression was noted for HDAC4, 8, and 10. HDAC4 was present in neurons producing histamine, CRH, oxytocin, AVP, orexin, serotonin, and noradrenaline. Noradrenaline was in common with HDAC10, which is also present in AgRP, POMC, and dopamine neurons. HDAC8 immunoreactivity was detected in the cytoplasm of almost all histamine neurons.[86] This differential expression of HDACs may provide clues as to their selective roles in neuronal function.

Class III HDACs are unique because they require NAD as a cofactor for their enzymatic activity. These enzymes, also known as the sirtuins (SIRT1-7), are structurally dissimilar to the other HDACs. Sirtuins are implicated in many critical cellular processes, including metabolism and aging.[87] The mammalian sirtuins are located in different subcellular areas. SIRT1, 6, and 7 are found in the nucleus; SIRT3, 4, and 5 are present in mitochondria; and SIRT2 is found in the cytoplasm. SIRT1, which is involved in lipid and glucose metabolism, stress responses, and aging and in the brain, is essential for B-cell lymphoma 6 protein (BCL6)-mediated neurogenesis.[88] One of the many functions of SIRT2 is to regulate transcription of sterol response element binding protein, which promotes cholesterol biosynthesis in neurons and is essential for myelin and steroid formation. In agreement with this fact, SIRT2 is present in particularly high levels in myelin-forming oligodendrocytes.[89] Both SIRT1 and 2 are present in human placenta, with lower levels in the amnion and choridecidua.[90] SIRT3 is expressed in brown but not white adipose tissue, where it interacts with estrogen-related receptor alpha to regulate responses to oxidative stress and calorie restriction. Also found in mitochondria, SIRT4 may be important for insulin downregulation in pancreatic beta cells.[91] SIRT5 is broadly expressed but not well understood. SIRT6 is present in most tissues throughout the body, and SIRT6 knockout (KO) mice die at about 1 month of age.[92] The final nuclear sirtuin, SIRT7, is present in the nucleoli; it interacts with RNA polymerase I and other DNA transcriptional factors. Given the importance of energy and metabolism for reproduction, these are clearly fundamental observations in understanding how the sirtuins are involved in reproductive processes.

HISTONE METHYLTRANSFERASES

Three histone N-terminal residues—lysine, histidine, and arginine—are subject to methylation, which generally leads to decreased access to DNA by transcriptional protein complexes. Histone methylation does not change the charge of histone tails but instead affects hydrophobicity, which influences the affinity of transcription factors for DNA. In addition, the histone methyltransferase (HMT) enzymes are more specific for certain histone modifications than are the enzymes associated with acetylation. Methylation can be graded; one, two, or three methyl modifications can be added to specific lysines, and up to two methyl modifications can be added to arginines. Histidines can only have one methyl modification. Also, methylation can be both activating and repressing, whereas acetylation nearly always enhances transcription.

Three enzyme families, containing over a dozen known specific enzymes, catalyze the addition of methyl groups, which are donated from S-adenosyl methionine (SAM) to histones: (1) enzymes with a highly conserved protein domain (so-called SET domains) found in chromatin regulators; (2) the DOT1-like proteins that methylate lysines; and (3) the members of the protein arginine *N*-methyltransferase family (PRMT), which methylate arginines. The specific enzymes are shown in Table 52.2. Many of these enzymes have targets other than histones and can act on transcription factors, DNA repair, and genome stability. Some of these enzymes can methylate proteins on chromatin other than histones.

HISTONE DEMETHYLTRANSFERASES

Two different enzyme families act to demethylate lysines: amine oxidases and jumonji C-domain containing iron-dependent dioxygenases. The arginine demethylases are less well known. Interestingly, it was only a few years ago that histone demethylation was noted. Previously, methyl groups on histones were thought to be permanent, similar to the situation for DNA methylation, which up until very recently was considered to be irreversible. There are specific DNA sequences that recruit methylation

TABLE 52.2 DNA Modifying Enzymes and the Associated Chromatin Enzymes and Modifications

Enzyme	Associations with Other Epigenetic Modifiers	Function	References
MeCP2	H3K27me3, H3K9me2, HDAC2, SIN3A/B, NCOR, COREST, REST, microRNA	Transcription repression and activation, nuclear organization, splicing, neuron development	93–95
MBD1	SUV39H1, HP1, H3K9me3, SETDB1	Transcriptional repression	96–98
MBD2	NuRD complex, HATs, PCAF, H3K9ac, H3K9me3, HDAC1	Transcriptional repression and activation, interactions with MeCP2	99–101
MBD3	NuRD complex, HDAC1/2	Development, stem cells	102
MBD4	SIN3A, HDAC1	DNA repair, transcriptional silencing, apoptosis, transcriptional repression	103
Kaiso (also known as Zbtb33)	Smrt/NCOR complex (HDAC3), H3K9	Transcriptional repression, cell division	104
Zbtb4	COOH-terminal binding proteins (CtBP) complex, microRNA	Transcriptional repression, bind DNA	105,106
Zbtb38	CtBP complex	Transcriptional repression, bind DNA	105
DNMT1	G9a, SET7, HDAC1, H3K9me2/3	Maintaining methylation	97
DNMT2	microRNA	Epigenetic inheritance, chromatin segregation, RNA processing	107,108
DMNT3a	H3K4me0	Methylation of imprinted genes in germ cells, de novo methylation, development	97
DMNT3b	H3K4me0	De novo methylation, development, immune function	97
DMNT3L	H3K4me0	Methylation of paternal imprinted genes in germ cells, de novo methylation	97
TET1	MLL	Imprinting, reprogramming germ cells, transcriptional repression, DNA repair	109
TET2	H3K4me3	Reprogramming germ cells, glcNAcylation	110
TET3	H3K4me3, microRNA	glcNAcylation	110
Gadd45	H3K4me3	DNA excision repair	111

Enzymes designated in lower case were identified in yeast.

enzymes. The best known are the Polycomb group (PcG) response elements (PRE) and the Trithorax group (TrxG) response elements (TRE). The Polycomb repressive complex 2 targets H3K27, while the TRE directs recruitment of enzymes to the fourth lysine on histone 3 (H3K4). Only PcG has been identified in humans.

Methyl marks may act in concert but also may exclude each other (Figure 52.4). For example, if H3K4 has three methyl moieties (me3), H3R2 cannot undergo dimethylation.[112] In another example, the histone demethylase JHDM1D binds to H3K4me2 or me3; after this is accomplished, it demethylates H3K27me2.[113] H3K4me3 generally enhances transcription, thus demonstrating that methylation is not always repressive. Combinations of methyl marks can have actions that differ from single marks; for example, H3K4me3 is active and H3K27me3 is repressive when they are present alone, but when both are present together, the cell is poised for transcription.[114] The demethylase enzyme for H3K27, KDM6A, can associate with other enzymes that work on the activating mark, H3K4. This may explain the combined actions of these two modifications.[38]

In keeping with the multiple actions of histone methylation enzymes, it is possible that the methyl modifications change the configuration of the chromatin and thus create new interactions by chromosomal looping.[115] Certain patterns of histone modifications are required for transcription factors to bind. Of particular interest is the fact that proteins of at least seven of the X-chromosome genes involved in X-linked mental disabilities have histone modification functions. These include: methyl CpG binding protein 2 (*MeCP2*), alpha thalassemia mental retardation

syndrome X-linked (*ATRX*) a DNA-dependent ATPase, lysine specific demethylase 5C (*KDM5C*, also referred to as *JARID1C*), another histone lysine demethylase PHD finger protein 8 (*PHF8*), the BCL6 corepressor BCOR, which forms complexes with PcG, the PHD finger protein 6, and the bromodomain and WD repeat containing protein 3 (BRWD3). Recently, a patient was diagnosed with both mutations in two of these genes (*MeCP2* and *ATRX*) and severe intellectual disabilities.[116] Clearly, mutations in these genes will have multiple downstream affects via faulty epigenetic maintenance.

Epigenetic Modifications to the DNA

Compared to the varied and multiple associations between histone modifications, proteins, protein complexes, and specific genes, epigenetic changes to the DNA itself are restricted to specific genes, and specifically to cytosine residues proximal to guanines. This is annotated as "CpG" to indicate C-phosphate-G, confirming they are next to each other as opposed to being CG base-pairs across the DNA strand. Because the change involves methylation of the fifth carbon residue of the cytosine, this is also often annotated as 5mC for 5-methylCytosine; it involves the transfer of a methyl group from a donor SAM (Figure 52.5). Cytosines can also be methylated when associated with the other nucleotides, adenine and thymidine, and even another cytosine, but this occurs much less frequently and is currently of unknown significance. However, a report suggests that this occurs more frequently in the brain compared to other tissues and, more importantly, increases as the brain ages.[117] A summary of the enzymes and associated proteins involved in DNA methylation can be found in Table 52.2.

CpG Islands

In the mammalian genome, the frequency of CG as compared with AT occurrences is less than that expected by chance. However, the frequency of CG increases notably in the promoter region of some genes, increasing to above 50%, as opposed to as little as 1% in the reading frame. This leads to the designation of a "CpG island". Cytosines that are methylated can undergo spontaneous deamination and thereby be converted into thymidines. This sequence change is believed to be the source of the evolutionary pressure that keeps CpG frequency so low. However, the addition of a methyl group to cytosines within the 5′ promoter region can also have profound influences on the transcription of the gene, with the canonical effect being gene silencing. This can occur via two different modes: a direct steric hindrance of transcription factor access to the DNA or a secondary recruitment of specific methylated DNA binding proteins, which in turn recruit additional repressor proteins and thereby effectively prevent transcription factor access and gene expression (Figure 52.6). Part and parcel of the ability of changes in CpG islands to regulate gene transcription is that many mammalian gene promoters either reside within or contain CpG islands, although some do not. Moreover, chromatin is often densely packed into nucleosomes, thus precluding gene expression. However, CpG islands are either found outside of nucleosomes or, if found within one, the associated histones contain marks that are conducive to, not repressive of, gene expression.[118]

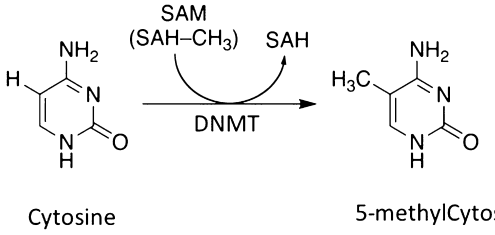


FIGURE 52.5 Methylated cytosine. Cytosines are the principal target of methylation in DNA. The methyl group is transferred to the 5-carbon of the cytosine by DNA methyltransferase (DNMTs) enzymes that use S-adenosyl methionine (SAM) as the methyl donor. Only cytosines found proximal to (not based paired with) a guanine are the target of methylation and this combination is referred to as CpG.

DNA Methylation Enzymes

In some instances, the methylation of CpGs is sufficient to regulate transcriptional activity, but often there is further recruitment of an additional set of proteins that both recognize and bind to highly methylated regions that can thereby further repress gene expression. These are referred to a methyl binding proteins and they come in a variety of forms (see Figure 52.6).

METHYL BINDING DOMAIN PROTEINS

The most famous gene member of the group of methylated DNA binding domain proteins (MBDs) is MeCP2, which was first identified as a critically mutated protein in Rett syndrome, a progressive neurological developmental disorder and the leading cause of intellectual disabilities in females.[119] Its location on the X chromosome is the basis for the sex bias in that males having their one and only copy of MeCP2 mutated rarely survive. MBD1, MBD2, MBD3, MBD4, and Kaiso are common methylated DNA binding proteins that recruit additional transcriptional repressors. These may further recruit DNA methyltransferases (DNMTs), resulting in more DNA methylation.[120,121] Some MBDs are also capable of both binding to specific sequences in nonmethylated DNA and still recruiting transcriptional repressors.[122] MBDs often also provide a platform for recruiting chromatin modifying enzymes that further alter transcriptional profiles (reviewed in Ref. 123).

In the most simplistic sense, methylation within CpG (mCpG) islands is correlated with and believed to be causally responsible for repression of transcription. However, as interest in the role of DNA methylation in

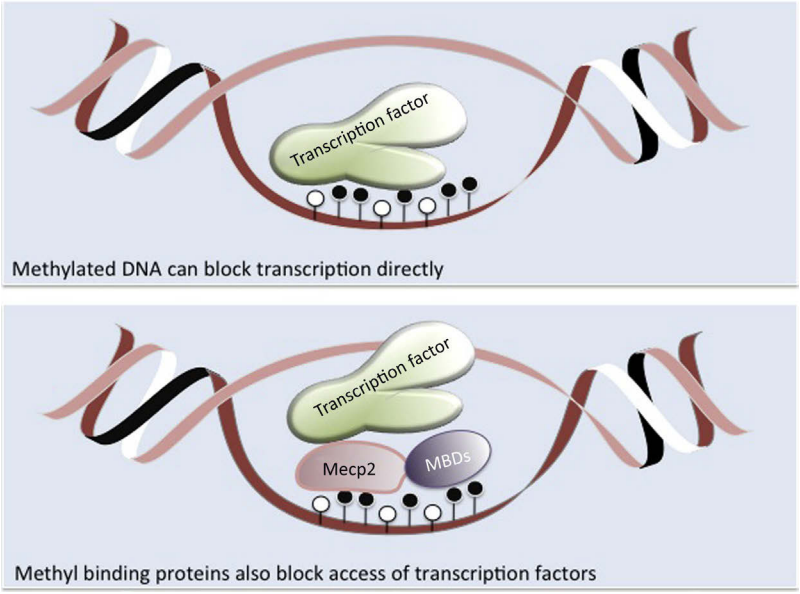


FIGURE 52.6 Impact of DNA methylation. The addition of a methyl group (in black) to cytosines or other residues can modulate transcription either by direct steric hindrance of transcription machinery associated proteins, blocking their access to the DNA, or indirectly by recruiting methyl binding proteins (MBD). One of the most well known is Mecp2, first identified as a critically mutated protein in Rett syndrome.

transcriptional regulation has grown, so has awareness that there is not a perfect correlation between mCpG and gene expression. One issue is that promoters typically have multiple CpG sites, and it is not typically the case that all of the sites are hypo- or hyper-methylated. Using a novel technology called sequence-tagged analysis of methylation patterns, Brenet et al.[124] conducted genome-wide analyses of densely methylated regions in distinct gene cassettes (i.e., transcription start site, first exon, introns and downstream exons). They found a high correlation in the amount of methylation in introns and downstream exons, but this was unrelated to the amount of mCpG in the promoter region. More importantly, the degree of mCpG in the promoter was variably associated with transcriptional regulation, whereas the methylation status of the first exon was more highly predictive, with greater methylation associated with transcriptional repression. In response to this and other discoveries, there is growing awareness of methylation of CpGs within the open reading frame (ORF) or within introns of some genes. Rather than mediators of transcriptional repression, these methylation marks are often more closely associated with past transcriptional history, meaning that after a gene has been transcribed there is an increase in intragene methylation, resulting in an inverse correlation between CpG methylation and gene expression. The amount of CpG methylation changes with aging and interestingly, generally increases in CpG islands but decreases at intragenic sites.[125]

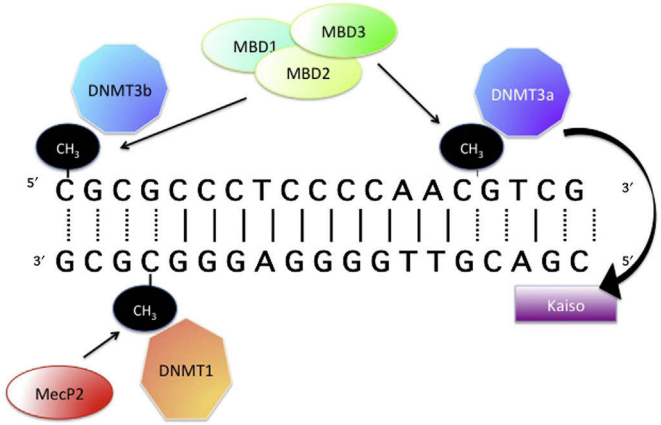


FIGURE 52.7 DNA methyltransferases (DNMTs). Two classes of DNMTs serve two different functions. DNMT1 maintains the previously established methylation pattern during DNA replication due to its ability to use hemimethylated CpGs as a substrate, thereby faithfully repeating the previously established pattern. DNMT3a and 3b are the so-called de novo enzymes as they can create new methylation patterns and their activity is modulated by environmental variables. DNMTs can also facilitate the recruitment of methyl binding proteins (MBD), which include MBD 1–3, Kaiso and MecP2 (which stands for methyl CpG binding protein 2 and is mutated in Rett syndrome). These methyl binding proteins can further recruit additional DNA or histone modifying enzymes, providing for cross-talk between DNA and chromatin.

DNA METHYLTRANSFERASES

The methylation of DNA is accomplished by a group of enzymes named for that function, the DNMTs. DNMTs carry out the addition of a methyl group to the five position of the cytosine pyramidine ring (Figure 52.5). Methylation can also occur on nitrogen of the adenine purine ring, but the functional significance of this is unknown. Mammalian DNMTs fall into three classes—DNMT1, DNTM2, and DNMT3—but only DNMTs 1 and 3 function as DNA-methylating enzymes (Figure 52.7). DNMT2 was misnamed and is not actually a DNMT, but instead methylates tRNA. The most abundant is DNMT1, and it is also

considered the maintenance DNMT. This is because it predominantly methylates hemimethylated CpG di-nucleotides, which are created during the process of DNA replication. Thus, with each cell division, the previous pattern of methylation on the single strand of DNA is matched on the second strand by the actions of DNMT1.[126] However, DNMT1 is also capable of de novo methylation, particularly in some mammalian cancers. De novo methylation is that which occurs outside the context of DNA replication; it is considered to be the form of epigenetic change that is responsive to the environment, hormonal signals, or other external modifiers. Most (if not all) of these methylation marks are removed during the wave of demethylation that occurs in the preimplantation embryo, thereby erasing the pattern of parental methylation. However, it is beginning to emerge that not all DNA methylation patterns are erased and reset at this time, and it has been suggested that novel DNA methylation can be inherited if epigenetic modifications fail to be reprogrammed. This so-called epigenetic inheritance is an active and controversial area of research with many questions remaining to be resolved.

DNMT3a and 3b are the de novo methyltransferases and are also critically important to early embryogenesis. Loss of either one results in severe developmental abnormalities, and the loss of both DNMT3 isoforms is embryonically lethal.[126] The first indication that DNMT3a and 3b serve different functions was in the distinct pattern of abnormalities and timing of mortality when each was mutated. The main difference between DNMT3a vs 3b is tissue distribution, with 3a being ubiquitously distributed and 3b much more restrictive after tissue differentiation, with some reports that it is found only in the testis, thyroid and bone marrow,[118] although others describe its presence in the brain.[127]

DNMT3L is an isoform of the DNMT3s that does not possess catalytic activity on its own but promotes the activity of its family members. It is predominantly expressed during development, being restricted to the testis and thyroid in the adult. However, during development it plays critical roles in the establishment of both maternal and paternal imprinting, inactivation of the X chromosome, and repression of transposable elements. In the nervous system, DNMT3L is expressed in the embryonic brain but is not found postnatally.[118]

DNA DEMETHYLATION

The covalent bond established when a methyl group is added to the 5′ position of cytosine is considered among the strongest in nature and is therefore difficult, if not impossible, to break enzymatically. Moreover, methylation patterns can be preserved during both meiosis and mitosis due to DNMT1-mediated maintenance during replication of the single strand, allowing for heritability of the epigenome marks. These facts contributed to a prevailing view of immutability of DNA methylation patterns. However, divergent and consistent evidence has challenged and modified this view to recognize that DNA methylation can be quite dynamic across the lifespan. In the simplest sense, the number of mCpGs can be passively reduced if there is an absence of active maintenance of methylation by DNMT1 during DNA synthesis. Thus, with each round of cell division, there will be a progressive loss of mCpG, with up to 50% lost for every turn through the cell cycle. As a result, DNA methylation is slowly and passively depleted, as is seen in the removal of methylation from the maternal pronucleus. However, this scenario only holds true for dividing cells and is not relevant to the many terminally differentiated cells, nor is it relevant to the two known naturally occurring waves of global DNA demethylation: one in the fertilized zygote and the other during primordial germ cell (PGC) development, confirming that indeed methylation marks can be removed.

In the zygote, the paternal pronucleus undergoes rapid demethylation shortly after fertilization, and much of this is due to oxidation to 5′-hydroxymethylated-2′-deoxycytodine, referred to as 5hmC (see below and Refs 128,129). PGCs, on the other hand, undergo genome-wide demethylation, erasure of all genomic imprints, and large-scale chromatin remodeling.[130] This restores the totipotency of the germ cell and is achieved within a single day.[131] However, there are also numerous other examples of more specific, focused, and modulated DNA demethylation, with emphasis in two areas: cancer cells and the brain. In the case of cancerous cells, inappropriate removal of suppressive methylation marks in the promoter regions of oncogenes is emerging as a critical event in dysregulation of cell cycle control.[132] By contrast, active demethylation in the brain is associated with normal events, such as learning,[133,134] but has also been implicated in the pathology of severe neuropsychiatric disorders involving major psychosis, such as schizophrenia and bipolar disorder.[135]

In all these instances, active demethylation is achieved in one of two general ways: either through oxidative 5mC demethylation, which is discussed below under ten-eleven translocation (TET)-family proteins, or through DNA repair. There are three separate ways in which DNA repair mechanisms can lead to demethylation: (1) removal of the 5mC and replacement with an unmethylated cytosine in a process called base excision repair (BER), (2) deamination of 5mC followed by BER, or (3) nucleotide excision repair (NER) to remove the 5mC (Figure 52.8). NER is a multistep process involving 20–30 proteins and is invoked when serious DNA damage has occurred, such as in response to ultraviolet irradiation. BER, however, is for small lesions, thousands of which occur daily in every cell

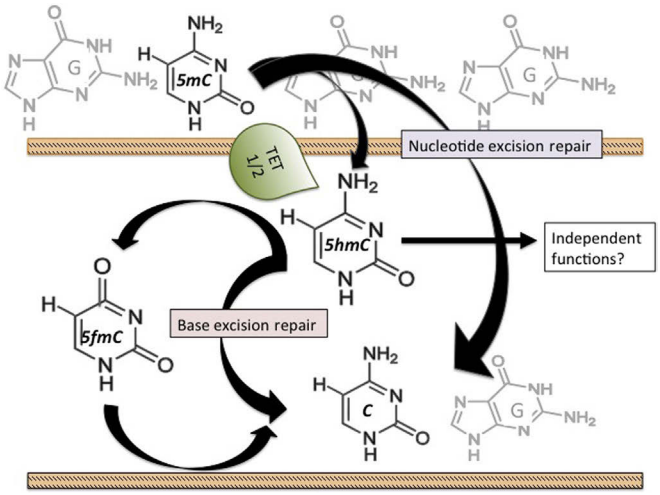


FIGURE 52.8 DNA demethylation. Demethylation of particular nucleotides can occur passively during cell replication due to failure of DNMT1 activity, but this process is generally slow and requires multiple cell divisions to significantly remove epigenetic marks. Active demethylation occurs predominantly as a by-product of DNA repair. The TET (ten-eleven translocation) family of proteins is emerging as major regulators of demethylation, which is achieved by first converting the methylated cytosine (5mC) to hydroxymethylated cytosine (5hmC) via oxidation. This substrate can then be further oxidized to 5-formylcytosine (5fmC) or 5-carboxylcytosine (not shown here). Both of these are then subject to base excision repair (BER) and return to an unmethylated cytosine (C), which can also occur directly from 5hmC. A 5mC can also be removed via the process of nucleotide excision repair (NER), a multistep process involving 20–30 proteins and invoked when serious DNA damage has occurred, such as in response to ultraviolet irradiation. Several intermediate steps in both these processes are excluded here for clarity.

and are induced by metabolic byproducts or exogenous agents. Understanding how these two processes can contribute to regulated demethylation, be it either active or passive, requires knowledge of the associated enzymes.

GROWTH ARREST AND DNA DAMAGE INDUCIBLE 45 As with many components of epigenetic regulation, growth arrest and DNA damage inducible 45 (GADD45) was initially characterized for its role in tumor growth. There are three isoforms of GADD45 (a, b, and g), and they all lack enzymatic activity themselves but induce demethylation by associating with effectors of the cell cycle and carrying out DNA repair. These enzymes are involved in both NER and BER and are responsive to signals that challenge cells such as stress, heat, and nuclear receptor-mediated hormones. One of the major questions regarding GADD45 activity is specificity—that is, how is it targeted to specific genes? Among the co-factors recruited by this protein are nuclear hormone receptors, including ERα, retinoid X receptor α, and retinoic acid receptor, and there is evidence that GADD45A may be involved in either demethylation or direct gene regulation induced by these and other associated nuclear receptors. There is also evidence suggesting that GADD45 interacts directly with DNMT1 and speculation that it may therefore prevent maintenance methylation from occurring during cell replication (reviewed in Ref. 136).

TET PROTEINS A new player has appeared on the scene as a potentially obligatory partner in DNA demethylation, the TET proteins. The emergence of these enzymes as important epigenetic regulators rests on their ability to convert 5mC into 5hmC via oxidation, and this substrate can then be further oxidized to 5fmC (5-formylcytosine) or 5camC (5-carboxylcytosine). Both of these are subject to BER. There are three members of this family in mammals: TET1, TET2, and TET3, all of which show high homology in their C-terminal catalytic domain. The major difference between the isoforms is their temporal expression pattern, tissue distribution, and the presence of a CXXC domain present in TET1 and TET3 but not TET2, which appears to enhance binding to CpG islands. Unlike Gadd45, where the strategy by which the enzyme is localized to sites of DNA methylation is unclear and may be indirect, both TET1 and TET3 are capable of direct association with CpG-rich regions, although controversial and ongoing studies also suggest interactions between the TET family of proteins and the methyl-CPG-binding domain (MBD) proteins, including MeCP2 (reviewed in Ref. 137).

Hints to the importance of TET protein activity are indicated by the differing amounts of 5hmC found across tissues and cell types. Although the level of 5mC is relatively constant across tissues (but varies between species), 5hmC levels vary widely, with the highest levels being found in specific cell types in the brain, such as the Purkinje neurons of the cerebellum.[138] Embryonic stem cells (ESCs) also have notably high levels of 5hmCs, and levels decrease as differentiation proceeds.[139] The elevation of 5hmC early in development with a decline as maturation proceeds was recently expanded to the frontal cortex.[117] A role for TET1 in maintaining the pluripotency of ESCs by maintaining hydroxymethylated DNA that is not recognized by DNMT1 has been proposed, but this notion has also been challenged. Both the TET proteins and 5hmC are found in gene-rich regions and are strongly associated with promoters; however, as with the presence of 5mC, the relationship to gene expression is not straightforward. As many genes appear to be repressed by the presence of TET proteins as are induced, further muddling the role of DNA methylation in gene expression. The differential effects of TET proteins are likely due to associations with repressive complexes such as SIN3A or the polychrome proteins. Ultimately, however, it appears that the high association of 5hmC and TET proteins at transcription start sites serves to maintain DNA methylation fidelity and to

largely keep CpG islands free of methylation and hence repression (reviewed in Ref. 140).

DNA DEMETHYLATION IN THE BRAIN

Several factors have converged to focus attention on the importance of DNA demethylation in the brain. These include a realization that although generally the brain is considered to consist of postmitotic permanently differentiated neurons, there are nonetheless high levels of key enzymes critical to both methylation and demethylation of DNA. When measured, DNA methylation of specific gene promoters is found to be mutable, changing in response to experience, learning, aging, or even hormones.[141–147] This, combined with a growing appreciation for the enduring impact of adverse early life experiences on adult brain and behavior, as well as the relative risk of developing disorders of mental health, suggest that something fundamental is imparted to the developing brain—a form of imprinting that is distinct from the genetic use of the term, although nonetheless a form of "memory". This memory may be the pattern and/or strength of synapses formed within particular brain regions, the rate at which new neurons are born in those select niches in which this process continues in the adult brain, or the expression pattern of key neurotransmitters, their synthetic and degradative enzymes, and the receptors that transduce them into altered neuronal activity. Expression levels of steroid receptors for both the gonadal and adrenal steroid classes are altered in discrete brain regions in response to epigenetic regulation.

The TET family of proteins is gathering interest in the nervous system because the adult mouse brain exhibits the highest levels of 5hmC when compared to other tissues, and this level progressively increases with age in at least three regions examined: the cerebellum, hippocampus[148] and frontal cortex.[117] The MBD protein, MeCP2, is also of interest regarding TET proteins. Assessment of genetically modified mice reveals an inverse correlation between MeCP2 and 5hmC in the cerebellum, suggesting that this so-called intermediate of hydroxymethylated C may actually be a stable mark that contributes to normal neurologic development. TET proteins are expressed in multiple brain regions, but a complete characterization has yet to be realized. A potential role for TET proteins in the evolutionary drive toward an increasingly complex brain seems feasible.[137]

Histone Modifications Interact with DNA Methylation

Although it is common for didactic purposes to separate the discussion of histone modifications from direct epigenetic changes on the DNA, in reality these two modes of modulation are intricately and mutually linked. For instance, members of the PcG place restrictive marks on the chromatin, which subsequently recruit enzymes that methylate DNA. Conversely, methyl CpG-binding-domain proteins bind to the DNA, then recruit chromatin modifying complexes that are usually associated with chromatin compaction and transcriptional repression.[124] Thus, there is coordinated regulation of histone and DNA modifications toward a common goal of either gene repression or expression. One universal principal that has emerged is that HDACs are essential for the recruitment of MBDs to specific genes. In primary neuronal cultures, MeCP2 can be acetylated by p300/CBP or deacetylated by the HDAC SIRT1. When *Sirt1* is removed in a nestin-cre mouse, the amount of brain-derived neurotrophic factor (BDNF), a target of MeCP2, is reduced. This demonstrates that there is a balance between HDAC and MeCP2 in the regulation of at least one gene, BDNF.[149]

As mentioned earlier, KDM5C is a histone demethyltransferase (HDMT) that reduces methyl moieties on H3K4. In addition, this gene resides on the X-chromosome, but it escapes X-inactivation. Thus, XX females have 1.5 times expression levels compared to XY males. The Y-chromosome contains a paralog of KDM5C, KDM5D, yet in male mice expression of this gene fails to completely compensate for the lower levels of KDM5C.[150] Mutations in KDM5C produce intellectual disability in males.[151–153] Recently, blood from a patient population revealed three target genes that might be downstream of KDM5C. The three genes had reduced levels of DNA methylation associated with their promoters, a situation that was not found in any of the 900 normal control samples. This relationship was also noted in patient brain samples.[154] These observations and many others strongly link histone methylation to DNA methylation.

Another intriguing study found that sex determination in the chicken is regulated by epigenetic marks associated with the *Cyp19a1* aromatase enzyme gene.[155] In many birds, exposure to estrogens prehatch can switch development of the genetically male gonad to an ovary. Blockade of estrogens has the reverse effect. In gonads of genetic males, estrogens decrease DNA methylation of the *Cyp19a1* promoter, which increases mRNA and thus enhances estrogen production. In addition, using chromatin immunoprecipitation (ChIP) for both H3K4me3 and H3K27me3, the authors found enhanced association of both modifications at the *Cyp19a1* promoter in male gonads after estrogen treatment.

One instructive way to think about the interactions between histones and DNA methylation is to consider the REST complex, which is implicated in a variety of brain disorders.[156] REST binds to the neuron-restrictive silencer element 1 (RE1) that is present on many neural genes, including genes for ion channels and neurotransmitter receptors. Once bound, REST acts as a scaffold for

other complexes, which in turn can recruit HDACs to the complex and CoRest, which recruits even more proteins, including HDAC1, 2, MeCP2, the HDMT LSD1, and others. Clearly, this complex is a hub of activity and interaction,[156] and in this manner the interrelationships between histones and DNA methylation are revealed.

OTHER EPIGENETIC MECHANISMS

One of the greatest surprises from the sequencing of the human genome was the realization that the number of genes was far less than expected (~25,000 at most), along with the discovery of vast quantities of so-called junk DNA. The central dogma of molecular biology is that DNA codes for mRNA, which codes for protein. However, only 2% of human genetic material functionally codes for proteins. Moreover, the genetic similarity between humans and other closely related primates, such as chimpanzees, is so great (95–98% homology) that it belies the tremendous divergence between the species. Multiple variables contribute to the divergence between such apparently closely related species, including pre- and post-translational processing. However, information encoded outside of canonical genes is also critical to their expression.

Noncoding RNAs

As the name suggests, ncRNAs are transcribed from DNA but are not translated into functional proteins. There are several types of ncRNAs that are grouped based on their size or function. The two main classes are the long ncRNAs (>200 nt) and the short ncRNAs, which includes micro (mi) transfer (t), and short interfering (si) RNAs. Many ncRNAs appear to have functions in housekeeping processes, such as protein trafficking and cellular stability. Another class is the regulatory ncRNAs, which may have discrete actions in certain tissues and/or at selected times in development; these are the ncRNAs involved in chromatin function. Here, we will focus on long ncRNAs and miRNAs because there is more work on these compared to other classes of ncRNAs.

Long ncRNA

The long ncRNAs are greater than 200 nt long and can be from intronic, intergenic, or genomic regions. Like mRNA, the long ncRNAs have a 3′ polyadenylated end and a 5′ cap, and they are transcribed by RNA polymerase II. Moreover, they often display enrichment for H3K4me3 at their promoter sites and H3K36me3 across their coding regions. These two marks denote active gene promoters and actively transcribed regions. The long ncRNAs also display marked differences from mRNA. For example, they are shorter than mRNAs and have fewer exons and introns. They are also less well conserved. The long ncRNAs are tissue specific and typically expressed at low steady-state levels. Because they are not translated into proteins, the long ncRNAs are found throughout the cell.[157]

The major role of long ncRNAs is to regulate transcription by altering enhancers, promoters, or other regulatory regions of the gene. This is accomplished by interactions with chromatin-modifying proteins. The best example of long ncRNAs that affect transcription

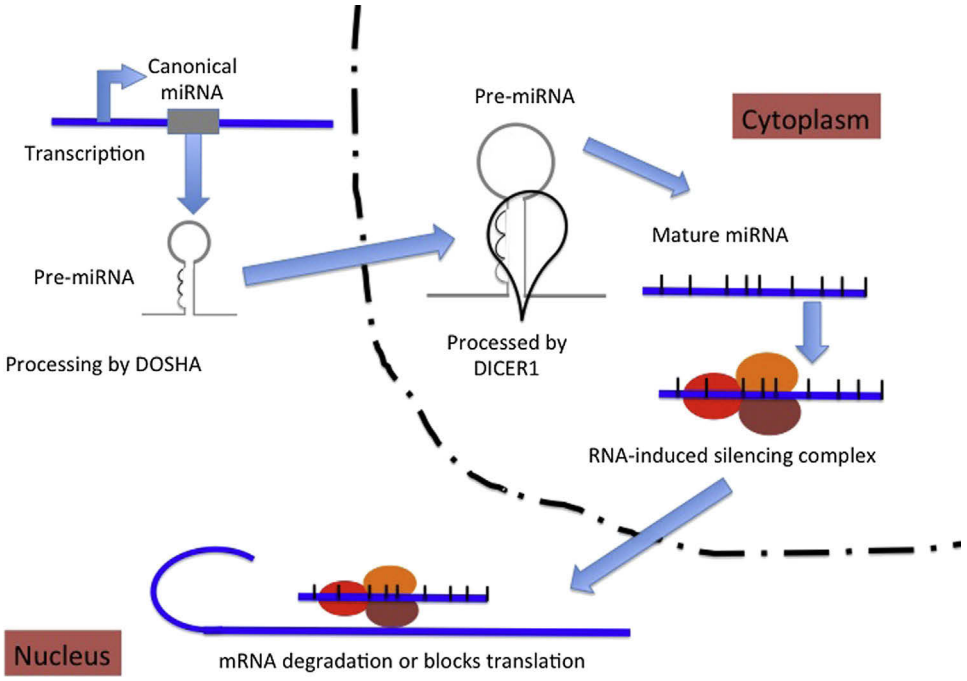


FIGURE 52.9 Processing of microRNAs. Transcription occurs in the cell nucleus where the pre-miRNA (microRNA) is processed by the enzyme Dosha. The pre-miRNA is processed in the cell cytoplasm by the DICER1 enzyme which clips the miRNA. The now linearized mature miRNA can act in a number of ways. As depicted here, it is bound to the RNA-induced silencing complex, then transported back to the nucleus where it binds to mRNA and blocks translation and/or degrades the mRNA.

are found in inactivation of the silenced X-chromosome. This process is detailed below. Essentially, the X-chromosome is silenced by the formation of repressed chromatin. The reverse function, promotion of active chromatin, can also be achieved by long ncRNA.[158] The long ncRNA *HOTTIP* acts with the myeloid/lymphoid (MLL) complex, which contains methytransferases; these then increase methylation of H3K4. Many of the actions of these ncRNAs are done in conjunction with the PRC2 complex.

ncRNAs can also regulate transcription by acting with transcription factors and coregulators. The steroid receptor RNA activator (*SRA*) is a *trans*-acting long ncRNA. *SRA* enhances the actions of liganded steroid receptors on their reporter genes.[159] SRA can associate with most steroid receptors, as well as the DAX-1 and SF-1 orphan receptors in the adrenals and may thus function in sexual differentiation.[157] Interestingly, *SRA* has been shown to produce a protein, SRAP[160]—the function of which is unclear, but it appears to interact with nuclear receptors. Another example of a long ncRNA that acts with steroid receptors is growth arrest specific 5 (*GAS5*). Some of the nucleotide sequence of *GAS5* is close enough to the glucocorticoid receptor (GR) response element that GR will bind.[161] Thus, when levels of *GAS5* are high, it can block the actions of GR. Finally, *CTBP1-AS* acts as an antisense to a corepressor of the androgen receptor (AR).[162] Long ncRNA also interact with and give rise to miRNAs. There are several examples of long ncRNAs that act as "sponges" to antagonize the actions of miRNAs.

Interestingly, brain sex differences in multiple regions can be attributed to Y- and X-chromosome genes.[163,164] Novel female-biased long ncRNAs in brain are associated with regions that surround genes that are known to escape X-inactivation. This observation suggests that sex differences in brain may be at least in part regulated by long ncRNAs.[163]

MicroRNAs

miRNAs are small (21–25 base pair) ncRNAs that regulate gene expression after transcription. These single-stranded RNAs target mRNAs for cleavage or translational repression. Humans have more than 1500 identified miRNAs so far (http://www.mirbase.org). They are produced from imperfect hairpin structures in long ncRNA precursors or from introns of noncoding genes. They are processed and produced in the cell nucleus by interactions between the enzymes DICER and DROSHA (Figure 52.9). In the cytoplasm, miRNAs interact by pairing with mRNA, the translation of which they block. miRNAs complex with ARGONAUTE and other proteins as part of the RNA-induced silencing complex. Hundreds of miRNAs have been identified in the past few years, and their targets include many genes and ncRNAs. They are important during development and interact with NOTCH signaling, *HOX* family genes, and SMAD proteins.[165] They can also regulate DNA methylation in mouse ESCs. In addition to developmental roles, many miRNAs are cell-type specific. The miRNAs interact with both DNA methylation and histone-modifying proteins and enzymes, representing another level of gene transcription regulation.

Multiple lines of evidence suggest that miRNAs are essential for embryonic neural development. Complete KO of *Dicer* is embryonically lethal, but *Dicer* KO in brain,[166] particularly in dopaminergic cells, is not lethal. Using a dopamine receptor-Cre to eliminate *Dicer* in the medium spiny neurons of the striatum leads to reduction of several miRNAs; mice showed reduced stride length and increased astrogliosis, but no increased neuronal death. Many miRNAs are present in brain, and miR-124 accounts for nearly half of all miRNA in brain.[167] MiR-9 is only found in the brain—both in embryos and adults.[168] The distributions of miR-128 and miR-124 are nearly exclusively in neurons.[169] Importantly, the REST complex may regulate brain miRNAs, including miR-9, 124, and 132, and these miRNAs also provide feedback on REST.[170]

In germ cells, X-linked genes are silenced during spermatogenesis, but X-linked miRNAs are not.[171] In addition, there are no known miRNAs associated with the Y-chromosome. In fact, more than one-third of the 77 X-chromosome-linked miRNAs are expressed in a testis-preferential or testis-specific pattern, suggesting that these X-linked miRNAs have functional roles in spermatogenesis.[172] In particular, X-chromosome-linked miR221/222 is essential for development of spermatogonia via its regulation of KIT protein and message.[173] Recently, the Bale laboratory has reported that male mice stressed prior to siring offspring have elevated levels of nine miRs in their sperm.[174] Their offspring were highly stress-reactive, but no data on the profile of miRs in offspring sperm were given. If any of the miRs affected in fathers were also modified in sons, this could suggest a role for miRs in multigenerational inheritance.

It has been demonstrated that overexpression of miR-132, a brain-specific miRNA, increases dendritic spine density and impairs novel object recognition.[175] MiR-124 is involved in normal spatial learning and social interactions.[173,176] Several miRNAs, including miR-132, have been implicated in the regulation of circadian clocks.[177] Variation in miRNA expression has been documented in the hippocampus of 24 different recombinant-inbred mouse strains as well as the parental strains.[178] Greenberg and colleagues[179] demonstrated a role for miRNAs in honeybee social interactions, suggesting that these molecules are important for brain function in multiple species. Clearly, miRs are an important additional level

of transcriptional control, which is only beginning to be described.

Another class of small RNAs is the Piwi-interacting (pi) RNAs; these are 28–33 base pairs and are found in male and female germ cells. PiRNA predominantly silence retrotransposons during embryonic germ cell development, but they are also emerging as regulators of nonretrotransposon sections of DNA and may be important in epigenetic modifications of transcription.[180] There are three known groups of piRNAs in mice, all in germ cells, at different developmental stages. Loss of piRNA function leads to spermatogenic failure.[181] These potent regulatory molecules can be transmitted via the cytoplasm of germ cells (sperm or oocytes) to the next generation and thereby maintain parent of origin epigenetic inheritance.[182,183]

LINEs and Short Interspersed Elements and Alu Elements

Embedded within the putative junk DNA are long stretches of repetitive sequences that Barbara McClintock identified as mobile genetic elements in her Noble prize–winning work on maize in the early 1900s. McClintock was ahead of her time in many ways, including proposing that in addition to being insertional mutagens, transposons might also influence the expression of surrounding genes and thereby serve as controlling elements. The latter view was not generally accepted. Instead, transposons were viewed as parasitic sequences that invaded host genomes for selfish purposes and ultimately did more harm than good. There is still good evidence that most transposons are selected against and purged from the genome,[184] but the interpretation that they are all bad has dramatically changed in recent years. Current thinking looks more toward host organisms exploiting the benefits of transposons as contributors to genetic variability and agents of evolutionary change. Transposed elements, which are distinguished from retro-elements in that they have been rendered incapable of further transposition due to mutation, also contribute as supporters of genome integrity via their role as components of centromeres and telomeres (reviewed in Ref. 185).

The characteristics that define mobile elements are also used to subdivide them into two classes based on size and mode of integration. Class I retrotransposons must first be transcribed into RNA and then integrated into the genome in a copy-and-paste approach; Class II DNA transposons integrate into the genome via a cut-and-paste approach, meaning they induce the breaking of chromosomes and insert themselves. Class I retrotransposons are further subdivided into those containing long terminal repeats (LTR) or non-LTR. Of the non-LTR, the long interspersed nucleotide elements (LINEs) are the most prevalent (reviewed in Ref. 186). DNA transposons, LTRs, and LINEs are 3–7 kb long, while short interspersed nucleotide elements (SINEs) are several hundred base pairs and usually do not exceed 600 bp (reviewed in Ref. 187). Analyses of 18 separate mouse strains categorized over 100,000 polymorphic transposed elements, finding twice the number that functionally altered gene expression as previously believed and determining that the majority of transmission was via the male germ line.[184] These emerging roles for LINEs and SINEs in regulation of gene expression and greater genetic variability also highlight specific roles in reproductive physiology and in the brain (Figure 52.10).

LINEs

LINEs are mobile genetic elements that are amplified via retrotransposition. They are the most widespread class of transposons in mammals, constituting ~20% of mammalian genomic content. LINEs are distinguished from LTRs in that LINEs do not have repeating sequences and are the only active class of non-LTR retroelements in the human genome. Most LINEs are no longer capable of retrotransposition, in large part because important 5′

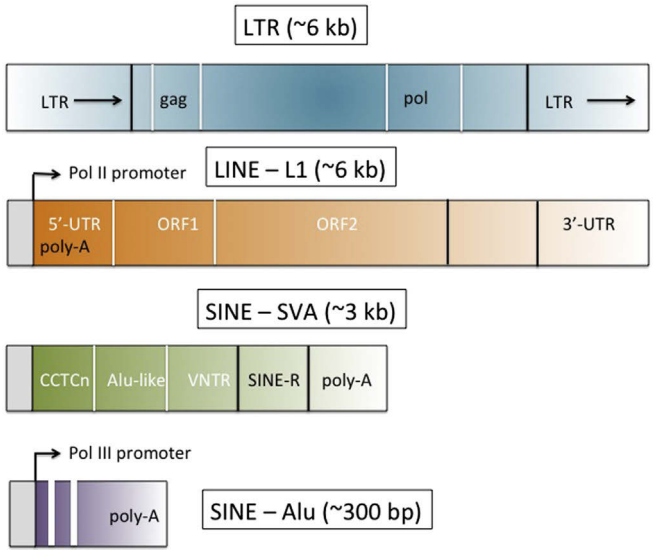


FIGURE 52.10 Transposons: LINEs, SINEs, and Alu Elements. Mobil DNA elements of the retrotransposon class are further subdivided into those containing long terminal repeats (LTR) or non-LTR. The LTRs and LINEs are both 3–7 kb long, while short interspersed nucleotide elements (SINEs) are in the several hundred base pair range and usually do not exceed 600 bp. LTRs are characterized by long terminal repeats at the beginning and ends of the insert. The proteins encoded by gag and pol are closely related to retroviral proteins. L1 is the dominant LINE and still capable of retrotransposition. The SINE category includes SVA, which is a composite of SINE-R, VNTR (variable non-tandem repeat) and Alu-like. Alus are primate specific SINEs and the most abundant in the human genome. LINEs include a polymerase II promoter site, while SINEs have a polymerase III promoter site as well. ORF1 and ORF2 are open reading frames.

regulatory sequences are lost upon insertion; nonetheless, there are almost 150 full-length active LI elements in the human genome and ~3000 potentially mobile LINEs in the mouse genome.[185] The average human genome contains ~80–100 active L1s[188] and up to 100,000 copies of human Line-1 are found in cells of the germ line.[189]

LINEs are considered autonomous because they code for proteins that they need for their own insertion (Figure 52.11). The most well characterized are L1 elements, which at full length are ~6 kb and contain an RNA polymerase II promoter in their 5′-UTR followed by two ORFs, referred to as ORF1 and ORF2. This is followed by a short 3′-UTR that ends in a poly(A) signal and an A-rich tail. ORF1 encodes a nucleic acid chaperone protein and ORF2 encodes an endonuclease and reverse transcriptase, both of which are essential for retrotransposition.[190] The LI RNA associates with the ORF1 protein and is then reverse transcribed by the ORF2 product to generate the cDNA, which is integrated into the genome. These same proteins can be used by the nonautonomous SINEs, as discussed below. When LI inserts, there are target site duplications at each end, and this can lead to the generation of pseudogenes.

L1 insertions occur in both somatic and germ cells. However, it is argued that from an evolutionary standpoint, the only significant transpositions are those occurring in the cells destined for the next generation.[191] In mammals, these include PGCs, which begin extraembryonically in the mesoderm, the germ cells proper, and very early embryos. Confirmation of active LI transpositions was confirmed via immunocytochemical detection of ORF1 protein in the cytoplasm of primitive spermatogonia, followed by Leydig cells in the developing testis, theca cells of the adult ovary, and the syncytiotrophoblast cells of the placenta. The common feature of each cell type is the production of androgens, and this close association was further confirmed by a temporal correlation between androgen production and LI ORF1 expression and a lack of ORF1 in steroidogenic cells of the mouse adrenal gland.[191] The mouse adrenal gland does not produce androgens due to the lack of 17-α-hydroxylase, precluding synthesis of C19 steroids. Most striking is the observed increase in ORF1 in the testicular Leydig cells at embryonic day 17.5, which is the height of the prenatal gonadal surge in androgen production. This is followed by a concomitant fall in both androgen synthesis and ORF1 production in the testes, shortly after birth, with blood levels remaining low until the return of androgen production at puberty. The results of this and other studies indicates that LI is actively expressed in at least five different cell types in the germ line, as well as in somatic cells involved in the production of androgens.

The importance of LINE retrotranspositions in somatic cells is a matter of debate, but the debate is heating up with the observation that the brain is a particular hot spot for these events. Stem cells in the brain are multipotent but limited and are thus referred to as neural progenitor cells, meaning they can become only neurons, astrocytes, or oligodendrocytes, depending on conditions.[192] Human fetal brain stem cells can give rise to neural progenitor cells that support retrotranspositions when transfected with an LI construct known to

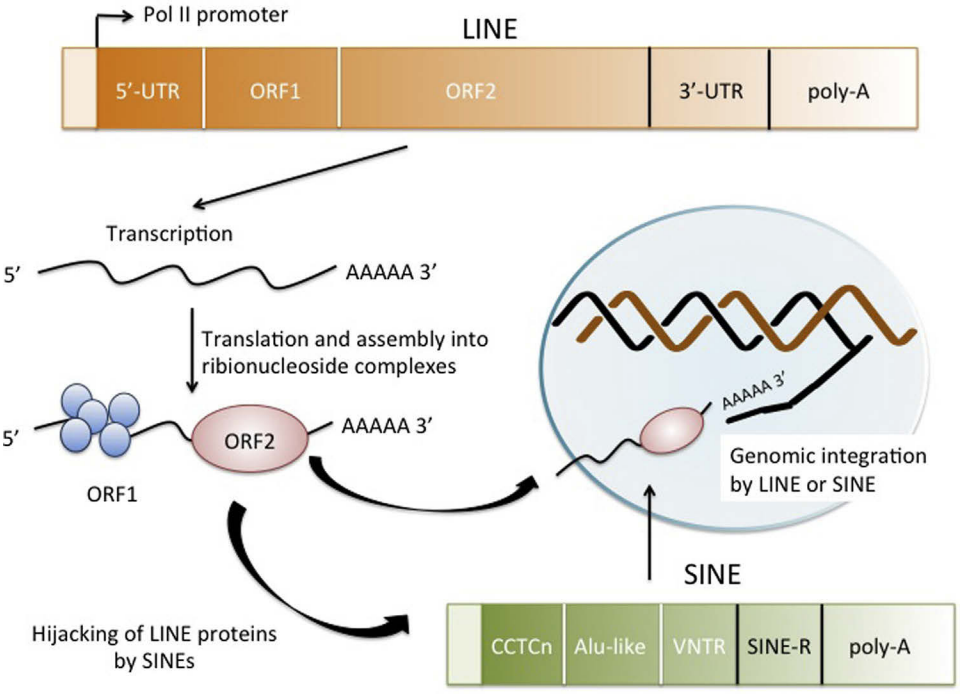


FIGURE 52.11 Retrotransposition. LINEs contain ORF1 and ORF2 (open reading frame 1 and 2), which code for chaperone proteins (ORF1) and an endonuclease and reverse transcriptase. Both sets of proteins are critical for self-replication and insertion as the parent DNA must be nicked and transcribed. LINEs are capable of self replication and insertion; SINEs are not but instead require transactivation by both the nucleic acid chaperone properties of ORF1 and the enzymatic activities of ORF2 in order to achieve successful retrotransposition.

be competent. Moreover, endogenous LI transcripts are detected in cultured human neural progenitors.[188] The activity of the LI promoter peaks during differentiation from stem cell to neural progenitor. The methylation status of the promoter is inversely related to retrotransposition efficiency, and bisulfite sequencing reveals that LI promoters in neural stem cells are far less methylated than those in skin. Further evidence of the predominance of L1 transpositions in brain is found in adults, where ORF2 transcripts are found at significantly higher levels in the hippocampus compared to the heart and liver of the same individuals. A more widespread survey of the brain found marked regional and individual variation in ORF2 copy number; however, regardless of specific region, the brain consistently outranked heart and liver.[188] Unfortunately, this study did not examine the reproductively relevant preoptic area (POA) and hypothalamus.

Hints that LI retrotranspositions might nonetheless be important to reproduction outside the germ line are found in the very few factors that associate with the L1 promoter, which of note includes the SOX family of transcription factors, with SRY being the prototype. SOX genes share a conserved high mobility group (HMG) box domain involved in DNA binding, as well as nuclear export signals that provide for rapid shuttling of SOX proteins in and out of the nucleus in response to dynamic cellular signals, such as calcium and cAMP.[193] A consensus sequence for SOX binding provides convergence for some of the multiple members of this family, with up to 11 different SOX proteins. There are two putative SRY-binding sites in the 5′-UTR of L1, and they are immediately 3′ to the CpG island.[188,189] Electromobility shift assay confirms the binding of the SRY HMG box to the L1 promoter, and luciferase reporter assays indicate a ninefold induction of L1 transcription by SOX11, with a smaller induction by SOX3 and a reversal of L1*luc* expression by SRY in the presence of SOX11. This is consistent with the absence of an activation domain in SRY.[189] SRY and related proteins may further modify LI transcription by associating with the only previously characterized transcription factor connected to the L1 promoter, YY1, and in that capacity contributing to the DNA binding and recruitment of additional regulatory factors. SOX2 associates with the LI promoter in human neural progenitor cells, but less so in mature neurons, consistent with the rate of retrotransposition. MeCP2 also associates with the LI promoter and, along with SOX2, is speculated to modulate L1 activity selectively in different neuronal cell types.[188]

In addition to the effects of SRY on L1 activity, there is evidence for L1 retrotransposition effects on *Sry* duplication and chromosomal translocation. Several rodent species have multiple copies of *Sry*, including the vole *Microtus cabrerae*, which is particularly striking for the presence of multiple polymorphic copies of the *Sry* gene in males and, unexpectedly, also in normal females.[194] In other species in which multiple copies, either polymorphic or monomorphic, of *Sry* are found, they are restricted to the Y chromosome of the male genome. The genus *Microtus* is noted for sex chromosomes of extreme size, so much so that they are called giant chromosomes, which is due to the presence of large blocks of constitutive heterochromatin. In *M. cabrerae*, the entire *p* arm and the proximal portion of the *q* arm of the X chromosome are inactive heterochromatin, while the entire Y chromosome is heterochromatic except for a tiny portion on the *q* arm, which is euchromatic. Embedded within the heterochromatic regions of both the X and Y are multiple clusters of *Sry* genes, with just a few sequences located in the euchromatic region of the Y. To date, all of these *Sry* genes have been found to be nonfunctional pseudogenes possessing internal stop codons, although clearly one functional copy exists on the Y chromosome, as sex differentiation is unremarkable in this species. Evidence of retrotransposon involvement in the remarkable expansion of SRY in this species is the prevalence of L1-mur3, a LINE element, and *Lx6*, an LTR, flanking both the 5′ and 3′ regions of the *Sry* pseudogenes in both X heterochromatin and Y euchromatin. An unanswered question is how *Sry* was transferred from the Y to the X chromosome, but one possibility is via ectopic homologous or nonhomologous recombination between LI elements on the two chromosomes. The high degree of sequence similarity in the *Sry* pseudogenes on the X and Y suggests that the transference was a relatively recent evolutionary event.[194]

SINEs and Alu Elements

SINEs are distinct from LINEs both in their length and by their dependence on the proteins coded for by LINEs that are required to actively transpose (Figure 52.11). In other words, SINEs are not autonomous but instead require transactivation by both the nucleic acid chaperone properties of ORF1 and the enzymatic activities of ORF2 from L1 or, in nonprimate species, other LINE elements. The same is true for other nonautonomous interspersed repeats, which include the primate composite repeat SVA (an acronym of SINE-R, VNTR, and Alu).

The Alu elements are deemed the most successful of all mobile elements, contributing almost 11% of the human genome with a copy number well in excess of 1 million. Alu elements are primate specific and dependent upon the only currently active primate LINE, L1. However, they also code for low levels of RNA that are transcribed by RNA polymerase III (as opposed to the more commonly utilized RNA polymerase II), and these RNAs contribute to retrotransposition. The body of an Alu element is about 280 bases in length, formed from two dimers separated by a short A-rich

region (Figure 52.11). The entire construct is flanked by direct repeats of variable length that are formed during insertion by duplication of the host sequences at that site. There is no terminator sequence for Alu transcription, so transcript size varies depending upon when the polymerase encounters a TTTT terminator sequence in the host genome. This and other variables make each Alu RNA unique, and hence difficult to track. Moreover, the RNA polymerase III activity is weak and dependent upon fortuitously landing near a strong regulator. Even if successful, most Alu transcripts are epigenetically silenced, resulting in very low levels of viable RNA. Nonetheless, some of the variable-sized transcripts coalesce into ribonucleoprotein particles that then associate with ribosomes, and presumably ORF2 protein. The Alu RNAs then utilize the purloined ORF2 to copy themselves at a new genomic site, using a process termed "target-primed reverse transcription". In this scenario, Alu elements do not require ORF1, only ORF2, to successfully transpose. This has important consequences (e.g., in testes, where L1 transcription is high), but almost all the RNA is not full length, mostly due to splicing.[195] However, sufficient ORF2 is made to allow for successful Alu element retrotransposition. Moreover, Alu elements can retrotranspose almost immediately after transcription, whereas LINEs require up to 24 h. These differences between Alu and L1 are speculated to underlie the higher prevalence of Alus in the genome and its greater association with disease states (reviewed in Ref. 196).

Primate-Specific SINEs and Alu Elements

Widely dispersed throughout the human genome, Alu elements modify gene expression via influences on polyadenylation, splicing, and RNA editing.[196] That this may be important evolutionarily is evident in the comparison of humans to our closet primate relative, the chimpanzee. Since humans' divergence about 6 million years ago, there have been ~2400 fixed Alu insertions in chimps compared to ~5000 in humans; in orangutans, there have been only ~250 over twice the length of time (12 million years). Notably, the rate of L1 insertions does not differ between humans, chimps, and orangutans; combined with the observation that Alu elements tend to insert in gene-rich regions and LI insertions are found more often in gene-poor regions, this leads to the impression that Alu elements have played an important role in shaping our genome and perhaps speciation. Younger Alu elements and LI do not display enrichment differences between gene-rich and gene-poor regions, suggesting the LI has been preferentially eliminated from gene-rich regions, possibly due to its longer length thereby subjecting it to more negative selection pressure. There is ongoing Alu element insertion in the human genome, with estimates of one new insertion event per 20 human births, leading to around one in every 1000 new human genetic diseases. The potential for tremendous diversity between individuals is evident when comparing the two completed human genomes, which are polymorphic for approximately 800 Alu elements. The basis of disease production by Alu insertions lies in its ability to disrupt a coding region or splicing signal, and while this is largely random, the X chromosome appears particularly susceptible to disease-causing insertions. While there is much interest in the deleterious consequences of insertions into germline cells, it is also evident that somatic cell insertions can lead to mutations that underlie cancer progression in particular.

Alu elements can regulate genes they land near in a variety of ways, but the effective integration into a gene is a relatively rare event. Alu elements are enriched in CpGs which, unless found in a CpG island, are usually methylated. It is speculated that ~25% of the CpGs in the genome originated as Alu elements, possibly including some CpG islands themselves. Alu elements consist of FRAM and FLAM, standing for *fossil right arm monomer* and *fossil left arm monomer*. They are each about 280 bp long, and these two, nonidentical monomers dimerize in a head-to-tail organization. In between the two is a variable-length, poly-A repeat. There are also transcription binding sites found within the Alu sequences, including the nuclear transcription factor family, of which steroid receptors are a member.

Alu subfamilies are categorized by their age into old, middle aged and younger, with the majority being in the younger category.[197] In the older families, spontaneous mutations have resulted in the creation of HRE's, or hormone binding elements, with the consensus binding site AGGTCA, which is recognized by the nuclear receptor super-family of ligand activated transcription factors including ER, PR, GR and retinoic acid, thyroid hormone and vitamin D receptors. Thus a reasonable approximation of an HRE recognizable by retinoic acid, thyroid hormone, vitamin D, progesterone, estrogen and glucocorticoids exists and these Alu sequences may serve as a cellular sponge, sequestering transcription factors away from viable gene promoters, or in some instances, influencing the regulation of gene transcription (reviewed in Ref. 197).

However, the data are stronger for an impact of Alu elements on posttranscriptional processing via modification of polyadenylation, alternative splicing sites, and changes to RNA editing. Alternative splicing involving Alu elements is referred to as "exonization" and is considered so widespread as to affect hundreds, if not thousands, of human genes. Exonization can produce novel splice variants, and some estimates suggest ~5% of all alternative exons are derived from Alu exonization.[197] One particular form of RNA editing is predominant in brain and involves two complementary Alu elements

forming a hairpin loop. The loop is then modified by double-stranded, RNA-recognizing enzymes, referred to as A-I editing, which results in the inability of the transcript to leave the nucleus, thereby short circuiting translation.[196]

There is an emerging relationship between Alu elements and the ER. The notion of a tightly restricted palindromic DNA sequence constituting an estrogen response element (ERE) and required for ER binding to gene promoters has been substantially revised in light of investigations outside of the chicken vitellogenin gene. Not only is there far more tolerance to imperfections in the ERE sequence than initially believed, there is also substantial interaction of ER with the DNA indirectly via other mediators such as c-Fos, c-Jun and AP-1, and probably others. Screening of ER interactions with human genes in yeast revealed the even more surprising finding that embedded within the estrogen-responsive breast cancer gene, *BRCA-1*, is a new subclass Alu element that diverged away from the original families and that acts as an estrogen receptor enhancer. The estrogen receptor enhancing component does not appear to be widespread, as the consensus sequence was not found in the four main subfamilies of Alu elements.[198] Independent analyses of MCF7 cells also found ER binding to repetitive sequences with Alu elements, in this case in the S subfamily, and that SRC3 was recruited to the same site in an estrogen-dependent manner.[199] Limited evidence suggests AR sensitivity of specific genes relevant to prostate cancer might also have been influenced by retrovirally introduced, LTRs.[200] While relatively modest in score, together these studies suggest that transposed elements may have been important contributors to steroid hormone sensitivity. How and whether this includes steroid signaling in the reproductive system remains to be determined.

Genomic Imprinting

Mendel's initial formulation of genetic inheritance did not include any differences between maternal and paternal alleles. We now know that this is not the case; over 100 genes have been shown to have parent-of-origin effects (Figure 52.12). An early demonstration of genomic imprints comes from attempts to make uniparental mouse embryos from pronuclei, which failed since such embryos do not develop normally and soon die. Interestingly, female-only (gynogenetic) and male-only (androgenetic) embryos fail in distinctly different ways. Gynogenetic embryos live until the 25-somite stage, with very little placental tissue. Androgenetic embryos have the reverse phenotype—well-developed placenta and stunted embryos.

The functions of many of the known imprinted genes are related to embryonic and placental growth; however,

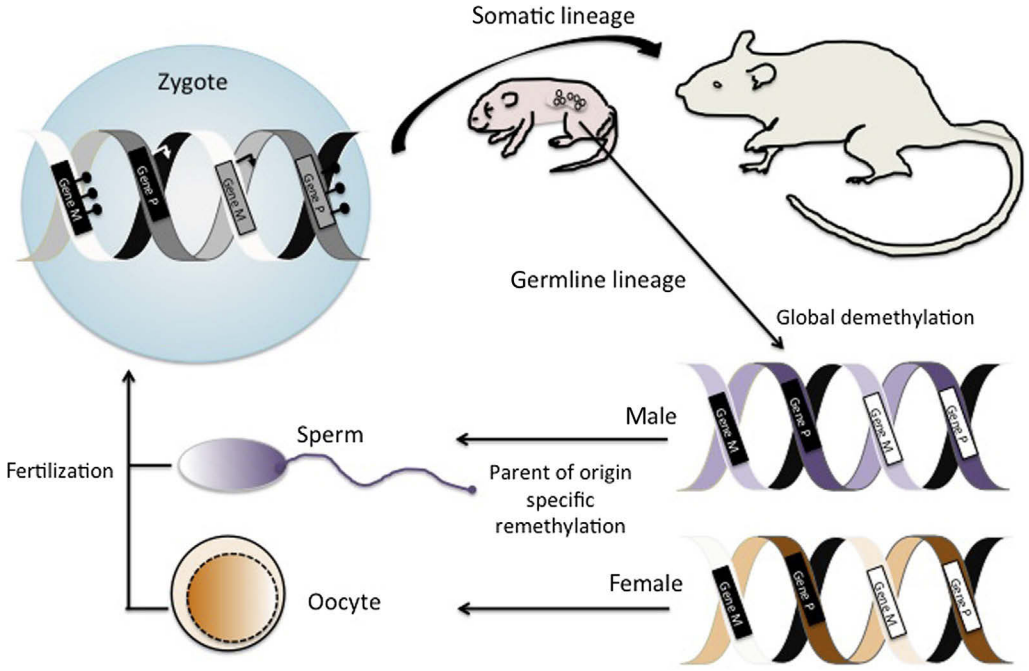


FIGURE 52.12 Genomic imprinting. Multiple genes are subject to the impact of imprinting, meaning the parent of origin determines the level of expression, with one allele being largely silenced systematically throughout the body. This is achieved with differential methylation of the allele on the chromosome from one parent. As illustrated in the figure, fertilization is followed by zygote formation and within the offspring genome maternal vs paternal copies of an imprinted gene may be silenced or expressed. The somatic lineage may not include expression of these genes, but the offspring's germ line will express the imprint during generation of germ cells.

others are involved in physiology, metabolism, and behavior.[201] The first imprinted gene identified was insulin-like growth factor 2 (*IGF2*), which promotes body growth. The receptor, IGF2R, can be thought of as growth-limiting, and its gene is silenced on the sire allele and expressed on the maternal allele.[202] Mutations in the maternal allele, therefore, have no effect since only the paternal copy is expressed. A more recently discovered imprinted gene is on the X-chromosome and encodes for RING finger protein 12 (RNF12), a ubiquitin ligase that acts to increase the dosage of X-inactive specific transcript (*Xist*) in cells with more than one X-chromosome.[203] The paternal copy of *Rnf12* is silenced.[204] In addition to in the embryo, *Rnf12* is also expressed in the mothers' milk-producing alveolar cells. In the few other cases of imprinted genes on the inactivated X-chromosome, including the recent studies showing that this occurs in the mouse brain,[205,206] it is usually the paternal X that is preferentially silenced. In addition to *Rnf12*, several other imprinted genes are involved in feeding. Expressed in the brain, *Peg3*, *Dlk1*, and *Gnasxl* regulate suckling behavior and are imprinted on the paternal allele.[207]

A variety of organisms experience imprinting: ranging from flowering plants to fruit flies to mice and to humans. However in vertebrates only mammals have evolved this mechanism and the genes subject to imprinting may not be conserved. In addition, there is evidence for tissue specific imprinting, as referred to above in embryonic vs placenta tissues. While less than half of imprinted genes have been examined in multiple tissues, of those that have been, only about 30% are specific and imprinted in only one tissue type.[207] Furthermore, some imprinted genes are only expressed at certain stages in the life cycle. The evolution of imprinting is not well understood, although the fact that it is widespread in plants and animals suggests some adaptive significance. The hypothesis put forward by Moore and Haig[208] suggests that the differences in mammalian parental investment affect imprinted genes. In general, maternally imprinted genes increase the size of the tissues that will become the placenta and paternally imprinted genes increase the size of the embryo. This leads to the "parental conflict" hypothesis in which the sire strives to utilize maternal resources and produce large offspring, at the energetic cost of the dam. On the other hand, the maternal genome is best served if the amount of energy provided by the dam is balanced with that provided to the embryo, and thus the imprinted genes regulating milk production and placental growth.

The mechanisms that underlie the process of parental imprinting are reminiscent of those described for X-inactivation, and this process uses many of the same proteins and enzymes. One difference between the two processes is that imprinting has a larger requirement for DNA methylation than does X inactivation. Many imprinted genes are found in clusters that also contain CpG-rich regions and which are only methylated on one of the two parental chromosomes. These clusters may serve as imprinting control regions (ICR), which act as either insulators or promoters for ncRNAs. In some cases, the ncRNAs have high affinity for trithorax proteins, which mediate H3K4 methylation.[209] Primary imprints in germ cells are erased and re-established during gametogenesis, and their differential methylation requires DNMT3a and DNMT3L. How maternal vs paternal ICRs are distinguished is not clear, but it might be related to location because maternal ICRs tend to be within transcriptional units and paternal ICRs are in intergenic regions. The imprinted genes persist during the early embryonic genome-wide demethylation. At this time, MBDs recruit additional silencing factors. The maintenance methyltransferase, DNMT1, is essential at this time, as is STELLA, a maternal factor formerly known as developmental pluripotency-associated 3 (DPPa3). STELLA binds to H3K9me2 to block the activity of TET3, which demethylates the genome globally.[210]

The importance of faithfully replicated, imprinted marks is illustrated by the consequences of when they are not. This can lead to Angelman syndrome or Beckwith–Wiedemann syndrome, with the former characterized by severe developmental delay, aphasia, and seizures and the latter by excessive growth and endocrine disorders. In vitro culture conditions can impact imprinting in preimplantation embryos, thus having consequences for in vitro fertilization (IVF). It has been proposed that children conceived with assisted reproductive technology (ART) are more likely to suffer from imprinting disorders, but other studies dispute this idea (reviewed in Chapter 45).

Keverne and colleagues have described the actions of the paternally imprinted mouse gene, *Peg3*, by using a *Peg3* KO mouse and manipulating the parent of origin of the null allele.[211] Dams pregnant with litters that were heterozygous for the null *Peg3* (from their sires) gained less weight during pregnancy; in addition, their pups were lighter weight and gained less weight than wild-type pups during the lactational period. After weaning, *Peg3*+/− females attained puberty at a later age and at lighter body weights than wild-type control females. Interestingly, when heterozygous dams mated with wild-type males to produce all wild-type litters, the dams gained less weight during pregnancy. Milk letdown was impaired in the dams and the pups were lighter than controls throughout, as well as after the suckling period. Thermoregulation was impaired in pups and dams that were *Peg3*+/−. These data illustrate that when the paternally imprinted Peg3 allele is disrupted, it reduces the size of the pups, and about one third died during the postnatal period. The expression

of *Peg3* in brain and placenta are also influenced by the nutritional state of the dam. After a 24-h fast during midpregnancy, *Peg3* mRNA increased in the hypothalamus but decreased in the placenta. The *Peg3* mutation affects a number of reproductive behaviors. In addition to maternal care, olfactory preferences for urine from estrous females and exploratory behavior are disrupted in males; in one mouse strain, maternal aggression was affected.[212,213] Some of these phenotypes are related to interactions between the oxytocin system, apoptosis, and *Peg3*+/− in mouse brain.[212,214]

X-Inactivation

X-chromosome inactivation is one of the best understood examples of epigenetic regulation of gene expression. In mammals, gonadal sex is determined by the sex chromosomes: XX in females and XY in males. This is the only pair of sexually dimorphic chromosomes. The human X-chromosome contains about 1000 genes, while the Y-chromosome contains only about one tenth of that number. Compensation for the lack of two copies of the X genes in males, as well as the potentially lethal overexpression of X-chromosome genes in the female, is accomplished by random inactivation of one X chromosome in each female cell. Thus, females are mosaic, having one X-chromosome that is euchromatic vs one that is heterochromatic. This essential difference makes females less vulnerable to genetic mutations on the X-chromosome because it is rare that both alleles would be mutated. However, when a female has one mutant copy, she will be mosaic for the expression of the protein it encodes and on a cell-by-cell basis will or will not have a phenotype.

As illustrated in Figure 52.13, the evolution of this system is thought to have started approximately 200 million years ago with the pair of autosomes that contained the *Sox3* gene, the likely predecessor to *Sry* in mammals.[215,216] In fact, this gene can substitute for *Sry* if it is inserted into the *Sry* HMG box and delivered as a transgene to XX mice.[217] The copy of *Sox3* on an autosome, which would become the Y chromosome, experienced mutations that resulted in the male sex-determining gene, *Sry*. Next, the region on the proto-Y gained male-specific functions and the genes nearby took on male-specific functions.[218] In fact, all the genes in this location now function in spermatogenesis. As a result of these modifications, the Y chromosome now passes only from fathers to sons. The pseudoautosomal region(s) (PAR) of the X- and Y-chromosomes are the only sections of the sex chromosomes that experience crossing over, and thus are common to both sexes.

To compensate for the loss of some of the essential genes on the Y-chromosome, the X-chromosome is hypothesized to have increased expression of a subset of genes that have paralogs on Y. These genes are designated as X-escaping genes. In humans, about 15% of the genes on the X-chromosome escape inactivation; in mice, that estimate is about 3%.[219] Other X-chromosome genes, without counterparts on the Y-chromosome, also escape inactivation. Of note, the actual rate of overexpression of these genes in XX females is less than twofold and is typically variable between individuals.[220] The final process in the evolution of the sex chromosomes was X-inactivation because it was essential to decrease expression of X-genes, which in a double dose might lead to lethality or other less deleterious effects. In contrast, it is obvious that overexpression of the escaping X-genes is also important. Turner syndrome is a fairly common (1:2000) aneuploidy condition with a complete or partial deletion of the second X-chromosome. In humans, despite

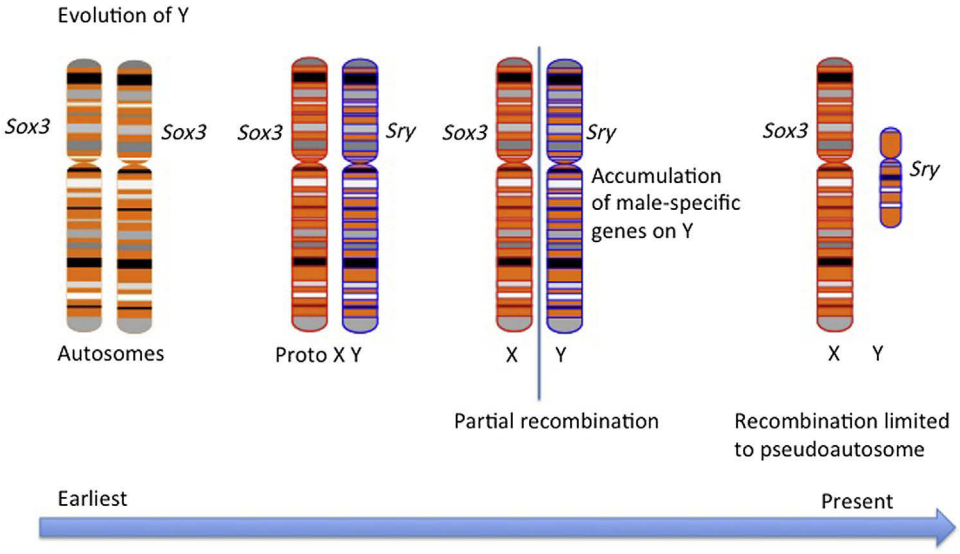


FIGURE 52.13 Evolution of the Sry gene and Y chromosome. Approximately 160–180 million years ago, the *Sox3* gene was located on a pair of autosomes in early mammals. This gene was the sex-determining gene. The next stage in this evolution was the modification of *Sox3* to give rise to the *Sry* gene only on the paternal autosome. Next, this autosome accumulated male-essential genes and a recombination block between the pair of chromosome established the pair as X and Y chromosomes. The number of genes on the Y chromosome was limited to those that had a sex-specific purpose leading to a reduction in the size of the Y chromosome. A portion of the X and Y still undergo limited recombination in the psuedoautosomal region.

the large number of live births, it is estimated that X0 is lethal in 99% of embryos with this mutation.[219]

Mechanisms of X-Inactivation

The long ncRNA, *Xist* (17-kb) spreads in *cis* along the X-chromosome (Figure 52.14). It is likely that LINEs, such as L1, assist in *Xist* spreading by serving an anchoring function.[221,222] In mice, genes that escape X-inactivation do not contain L1 repeats nor do they recruit *Xist*.[223] In addition, binding and propagation of *Xist* may require Yin Yang 1 (YY1), a protein that is suspected to bridge *Xist* RNA to its own locus.[224] The ncRNA *Tsix* is the antisense of *Xist* and spreads along the active X-chromosome, enhancing gene expression and thus maintaining accessibility to these genes. It is thought that *Xist* and *Tsix* are formed from the same RNA strand, which is cleaved by the RNAi enzyme DICER. Finally, *Xce* is an enhancer for *Tsix*. In addition, during random inactivation, both *Xce* and *Tsix* are thought to affect the choice of the inactive X (Xi) vs the active X (Xa). Mutation studies show that *Tsix* activity is required to suppress *Xist* on the Xa.[225] However, in male embryos, *Tsix* mutation is not lethal, suggesting the importance of factors other than *Tsix*. Data from male embryos suggest that the HMT *Eed*, one of the PcG genes, may be one of the other essential factors.[225–227] This enzyme trimethylates H3K27me3, a mark of repressed chromatin.

The PcG includes two repressive complexes: PCR1 and 2. These proteins are essential for targeting genes to be silenced by producing histone modifications. They are also essential for maintaining the silenced X. The essential PCR2 genes include *Eed*, *Ezh2* (also a HMT), *RbAp48* (includes a histone-binding domain), and *Suv12* (interacts with DNA via a zinc finger). All H3K27 methylation (mono, di, and tri) requires the PcG2 proteins. When *Xist* spreads over the Xi, a short ncRNA called *RepA*, within the *Xist* region, is part of the process that recruits PcG2 via *Ezh2*. A series of chromatin modifications result in silencing of nearly all the genes on this chromosome. The silenced chromosome cannot be reversed, and it is stably maintained in a silenced state through mitotic divisions. The first of these histone modifications is the recruitment of the repressive chromatin modification, H3K27me3. Other marks, H3K9me3, and a histone variant, macroH2A1, are also enriched on the Xi. This histone variant and de novo DNA methylation on promoters of Xi genes mediate long-term silencing. It is also likely that *Ehmt*, the euchromatic histone lysine-*N*-methytransferase 2, acts on H3K9 to promote DNA methytransferases, which also result in gene silencing. However, the recent discovery that DNA methylation need not be permanent may shed new light on long term X-inactivation.

Genes that Escape X-Inactivation

Clearly, an important class of epigenetically regulated genes are those on the inactivated X-chromosome (Xi) that escape inactivation. In humans, 15% of the genes on Xi are not inactivated; in the mouse, a study suggests that significantly fewer (3%) of all genes escape inactivation.[228] It is not known why Xi is more complete in mice than in humans. It is possible that the placement of the centromere, in the middle of the human X but at the end of the mouse X-chromosome, could be important. One hypothesis is that the centromeric heterochromatin in the human X may impair *cis*-spreading of *Xist*.[229] The locations of these genes in the human X are in clumps, containing up to 13 Xi-escaping genes, but in mice they are more spread out over the entire chromosome. The ability of the Xi-escaping genes to avoid X-inactivation is intrinsic to the DNA sequence and not dependent on their location on the X-chromosome. This was demonstrated by insertion of bacterial artificial chromosomes containing *Kdm5c* (also known as *Smcx* and *Jarid1c*), a gene that escapes X-inactivation into active locations on

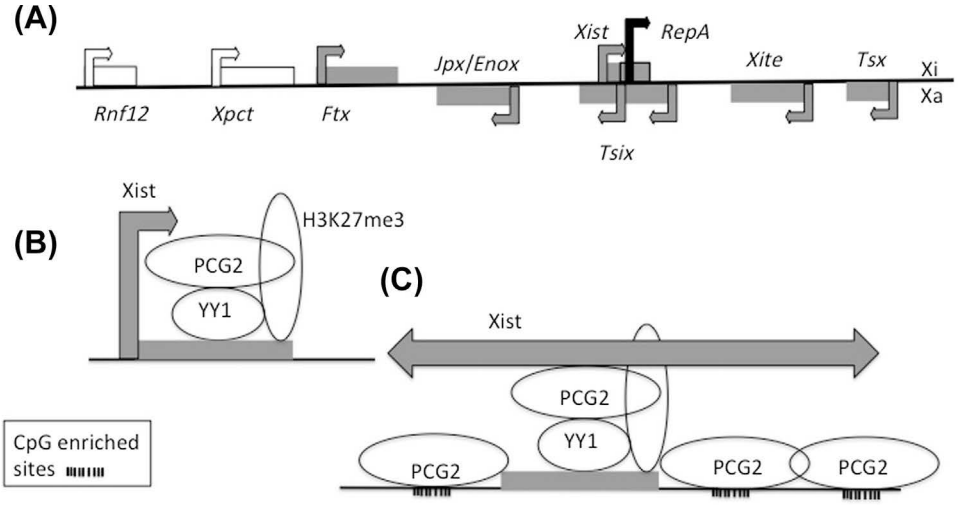


FIGURE 52.14 The control center and initiation of X chromosome inactivation. (A) The X-inactivation center consists of multiple genes (*Rnf12* and *Xpct* in white) and lncRNA, including *Xist*, *RepA*, *Tsix*, *Xite*, *Jpx/Enox*, *Ftx*, and *Tsx* (in gray). Xi = silenced X-chromosome. Xa = active X-chromosome. (B) When X inactivation is initiated, Xist recruits the polycomb group 2 complex (PCG2) and yinyang1 (YY1). YY1 bridges the repressive H3K27me3 modification. (C) As Xist expression spreads over the Xi, the PCG2 targets CpG enriched sites to silence transcription.

the X; in all locations, expression was present.[230] One clump of Xi-escaping genes present in both species is the PAR, which is the only region subject to crossing over between the X- and Y-chromosomes. The genes on the X-PAR have homologs on the Y; in addition, some of the Xi-escaping genes in other regions of X also have Y paralogs. Two such genes are *Kdm5c* and *Kdm6a* (also called *Utx*), and both produce histone demethylases that act at H3K4me3 and H3K27me3. Interestingly, H3K4me3 is an activating mark, whereas H3K27me3 is repressive and is important for X-inactivation. The Xi-escaping genes are devoid of *Xist* and contain nucleosomes with modifications that promote transcription, specifically H3K4me2, total H3, and H4 acetylation. Plus, their CpG islands are not methylated. In addition, the chromatin between active and inactive genes is associated with the CCCTC-binding factor, an insulator protein that prevents the spread of heterochromatin by blocking promoter and enhancer interactions.[231] LTRs are also depleted at Xi-escaping genes that have AT-rich motifs.[232] One aspect of these genes that has not received much attention is whether these genes have some unique function(s) or evolutionary roles that give rise to their escape. Lastly, inactivation of one of the two homotypic sex chromosomes is not universal. In chickens, female sex chromosomes are ZW and male sex chromosomes are ZZ, but the issue of dosage compensation would still be predicted to exist. In contrast to mammals, far fewer genes are subject to inactivation on the Z chromosome than would be expected, with most Z genes being expressed at 40–50% higher rates in males.[233] The presence of CpG islands in the promoters is the best predictor of whether genes are subject to dosage compensation on the Z chromosome, with long interspersed nucleotide elements, or LINEs being the next best predictor.

Early Preimplantation X-Inactivation

There are several reasons to hypothesize that the maternally derived X-chromosome, Xm, and the paternally derived X-chromosome, Xp, are not equal. One indirect piece of evidence is that Xp0 and Xm0 embryos grow at different rates (Xp0 embryos grow slower[234]). In addition, embryos with two Xm chromosomes are lethal.[235,236] However, whether the Xp is predisposed to inactivation, the Xm is resistant to inactivation, or both processes occur is not yet clear.

The process of X inactivation occurs twice during the female (XX) life cycle. The first time is in the early cleavage stage embryo (4–8 cell stages in the mouse). At that time, silencing is not random and only the Xp is inactivated. By the two-cell stage after fertilization, the Xp is expressing *Xist*, which leads to RNA pol II exclusion. Two active marks are suppressed: by the 8-cell stage, H3K9 is deacetylated and H3K4 is demethylated. At this point, X-genes on Xp decrease in expression and the PRC2 complex along with H3K27me3 appear on the Xp. By the 16-cell stage and later, the Xp remains inactive in the tissues that will become the trophoblast, but the Xp is reactivated in the inner cell mass (Figure 52.15). Then, just after implantation in most mammals, in the somatic cells but not in the germ cells, one X-chromosome is randomly inactivated. Marsupials are the exception: at both stages, only the paternal X-chromosome is inactivated. The question of how the Xp and/or Xm are marked such that only the Xp is inactivated at the four-cell stage likely involves the actions of the recently discovered TET enzymes discussed above. TET enzymes demethylate DNA by converting 5mC into 5-hydroxymethyl-cytosine (5hmC), 5-formlycytosine (5fC), and 5-carboxycytosine (5caC). Further evidence for functionally significant differences in the Xp vs Xm is found in individuals with sex chromosome aneuploides. Girls with Turner syndrome (XO) may inherit only one complete X-chromosome, which

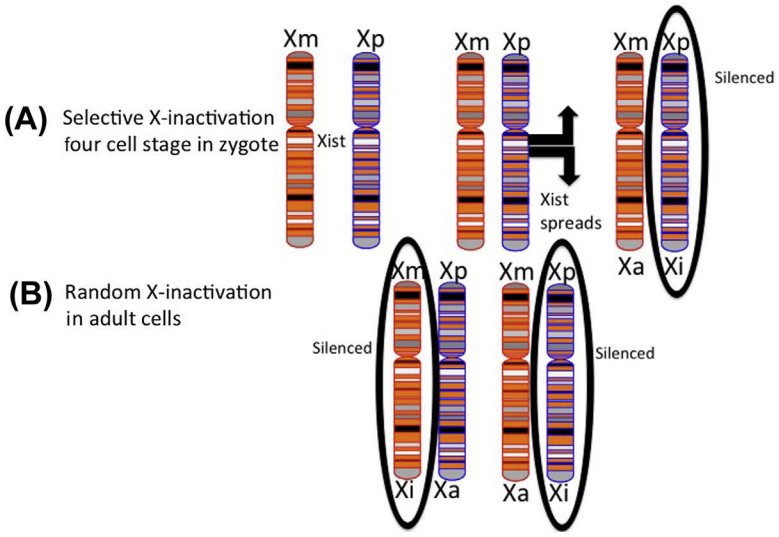


FIGURE 52.15 Selective and random inactivation of the X-chromosome. (A) Shortly after fertilization, the paternal copy of the X chromosome is selectively inactivated in every cell in the zygote and/or developing embryo. (B) In adults, X-inactivation is a random event. Either the maternal or the paternal X chromosome can be silenced. In both cases, the long ncRNA Xist is expressed and this leads to silencing.

can come from either their mother or their father. Skuse explored whether there was a parent-of-origin effect in X-monosomic females. He found better social and cognitive abilities in the females with a paternally derived X-chromosome. Several laboratories have reported brain differences between XO individuals with a paternal vs maternal X, including cortical thickness and regional gray matter volumes.[237,238] In contrast, Klinfelter's syndrome males (XXY) who inherit a second X from their fathers have more severe impairments of motor, speech, and language skills than males with two maternally derived X-chromosomes (reviewed in Ref. 239). These findings speak to the intimate interplay between imprinting and escape from inactivation of select genes on the X-chromosome.

Dosage Compensation

Both vertebrates and invertebrates have evolved systems of dosage compensation to balance expression of sex chromosome genes between the sexes.[229] Birds (ZZ) and mammals (XX) with homogametic sex chromosomes accomplish compensation by inhibiting expression of genes on one of the two identical chromosomes—X or Z for mammals and birds, respectively. In chickens, the Z chromosome is associated with ncRNA recruitment of H4K16ac; this mechanism is also found in the fruit fly, *Drosophila melanogaster*.[233,240,241] In *D. melanogaster*, the number of X chromosomes determines sex. In these animals, amplification of genes on the single X accomplishes dosage compensation. The male specific lethal (*Msl*) ncRNA is part of a larger protein complex (referred to as MSL), which contains recognition sites targeted by the histone acetyl transferase subunit, MOF. MOF is a member of the MYST family of HATs and acetylates H4K16 to enhance gene transcription.[242] In the nematode, *Caenorhabditis elegans*, males have one X-chromosome and hermaphrodites have two, both of which are partially repressed.[243] In placental mammals, not only is one X-chromosome inactivated, there is also upregulation of X-genes. This may be caused by a combination of factors, including: (1) histone acetylation, (2) Pol II enrichment on two activating modifications,[244] and (3) less rapid degradation of transcripts from X.[245] In marsupials, X-inactivation is not a random event. Only the paternal X is inactivated. This occurs without *Xist*. Instead, a different ncRNA, *Rsx*, triggers silencing. These examples demonstrate the variety of ways that different animals have evolved to compensate for dosage differences.

EPIGENETICS AND THE PHYSIOLOGY OF REPRODUCTION

The centrality of epigenetics to reproduction is self-evident in the reprogramming of the single-cell zygote into a fully differentiated individual, which itself is capable of reproduction. Every cell must undergo some degree of epigenetic programming in order to obtain its ultimate phenotype, and much of this is developmentally programmed. But there is also an emerging conceptualization that the early environment, meaning in utero, plays an important programming role in determine adult health and disease and that much of this is coded in epigenetics.[246]

Multiple fields have converged at more or less the same time on the concept of the enduring effects of the gestational environment, with the biggest impact coming from the Barker hypothesis,[6] which proposed that fetal undernutrition and restricted growth leads to increased coronary heart disease in adulthood. However, there is also less-celebrated work showing that prenatal stress impacts adult reproductive behavior in male rodents.[247] More recently, there has been careful attention to the enduring impact of exposure to introduced toxins such as nicotine, alcohol, drugs of abuse, and manmade chemicals such as plasticizers. In the latter case, there is evidence that epigenetic changes in response to so-called endocrine disrupters can manifest in the germ line and be transmitted cross-generationally,[248] a concept that is also emerging in the fields of fetal nutrition and prenatal stress. This topic is covered in detail in Chapter 45.[249] Appreciating how a fetal exposure event can endure across multiple generations requires an understanding of timing and origins of reprogramming of the germ cells, which occurs during fetal development and thus creates a window of opportunity, or vulnerability, for imprinting the environment throughout and beyond an individual's lifespan.

Epigenetics during Germ Cell and Early Embryo Development

As discussed above, there are two life-critical prenatal demethylation events: (1) the reprogramming of PGCs in order to restore totipotency; and (2) the removal of many, but not all, of the epigenetic marks in the newly created zygote (Figure 52.16). For the PGCs, this process occurs during their migration into the genital ridge, which is at ~E10.5 in the mouse, coinciding with the onset of *Sry* expression that will direct the formation of the testis in the male. The reprogramming of the genomic potential of PGCs is considered unique among epigenetic processes, as it is a complete reversal involving extensive remodeling of the chromatin, erasure of all imprinting, and the removal of epigenetic marks, including the active removal of DNA methylation. PGCs are derived from epiblast cells, meaning that they come from cells already embarking down a differentiation pathway, an event in the mouse that occurs at ~E6. This is followed by migration along the genital ridge before taking up residence in the now sex-specific gonad by day 12.5.[250] By embryonic day 13.5, the restricted population of PGCs

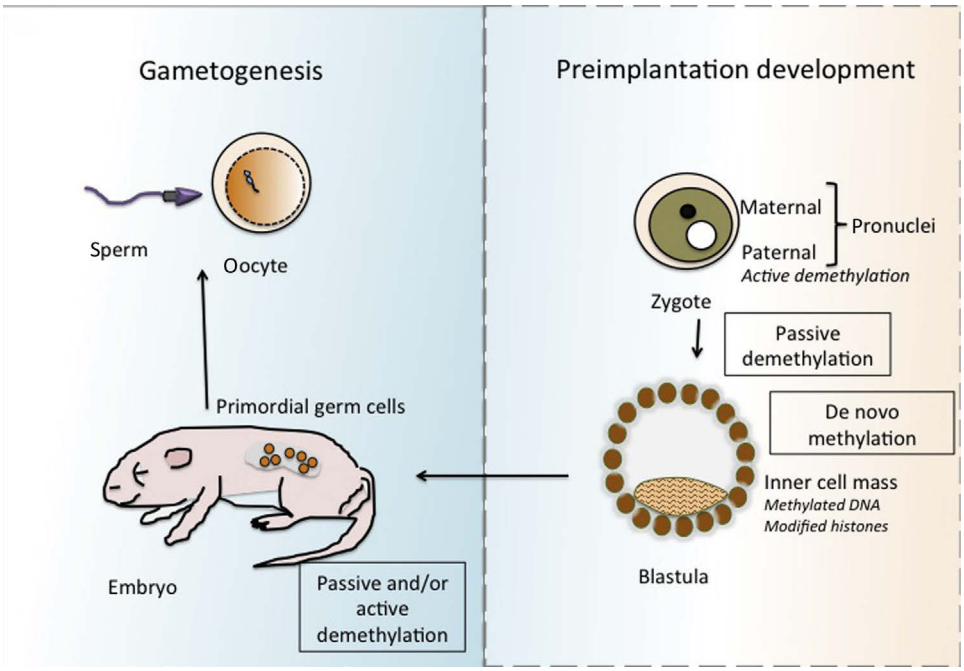


FIGURE 52.16 Two phases of global demethylation. There are two critical prenatal demethylation events, the reprogramming of primordial germ cells (PGCs) during gametogenesis in order to restore totipotency, and the removal of many, but not all, of the epigenetic marks in the newly created zygote. Both involve a combination of passive (secondary to cell division) and active demethylation but the timing and overall contribution of each process varies depending on sex.

either undergoes mitotic arrest in males or meiotic arrest in females; therefore, the deprogramming of the previous cell fate and reprogramming for totipotency, including reactivation of an inactive X chromosome if present, occurs rapidly and is considered the epigenetic nadir of mammalian development. Recently, it has emerged that there are demethylation-resistant genomic compartments that include transposable elements. Moreover, the rate and timing of remethylation differs in male vs female PGCs. Collectively, the results indicate that sex differences are present in methylated CpG islands on autosomes in the PGCs after gonadal sex determination. This occurs largely through increased CpG methylation in male germ cells while the X-linked genes, in both sexes, remain resistant to de novo methylation.[251] The mechanisms by which resistance to both demethylation and de novo methylation are conferred is unknown, but plausibly they involve DNA binding factors that block or recruit specific methylation enzymes.

Given the essential role of DNA demethylation in the PGCs and in the zygote, the presence and activity of TET family proteins during these processes has been of interest. PGCs do not express TET3, but they do express TET1 and 2 and therefore may also increase 5hmC. TET-mediated demethylation is emerging as one component of the global demethylation that occurs in both migrating PGCs and the zygote, but there are clearly other players as well, including the cytidine deaminase pathway and passive demethylation consequent to cell division.

An important question is: What is the function of global demethylation in PGCs—meaning, what purpose does it serve? One initial and primary purpose is to allow for activation of key genes required for PGC development, and these genes are normally tightly silenced by promoter methylation and attendant chromatin changes so as to avoid ectopic expression and possible tumor formation. A second theorized purpose is the removal of so-called epimutations, which are inappropriate epigenetic changes that would be detrimental if inherited by the next generation. Third is related to the expression of transposable elements (discussed in detail above), which are largely considered to be harmful and are therefore usually epigenetically silenced. Global demethylation runs the risk of allowing these harmful elements to awaken and even to propagate by further translocations. Paradoxically, there is evidence that global demethylation allows for additional precise repression of transposable elements, possibly involving small RNAs expressed by the elements themselves.[252]

The pervasive and complete removal of prior epigenetic marks in PGCs is distinguished from the other wave of global demethylation that occurs in the zygote in that the latter retains imprints, some DNA methylation, and polycomb-mediated chromatin modifications. 5mC in the paternal, but not the maternal, pronucleus is oxidized to 5hmC and TET3 is the key enzyme catalyzing 5mC into 5hmC in the paternal genome (i.e., paternal pronuclei) of developing zygotes,[128,129] with that TET3 coming from the maternal genome. The maternal pronucleus appears to be protected from demethylation by STELLA protein,[129,253] which specifically binds to H3K9me2.[210] In this way, a critical differential regulation of DNA methylation of the paternal vs maternal genome is established.

There is emerging consensus that there are mechanistic similarities between the two waves of global

demethylation, but there are distinctions as well—many of which are only recently emerging. One example is the role of TET3, which is the only TET family member expressed in zygotes, and the wave of cytosine demethylation (5mC) is accompanied by an increase in hydroxymethylated cytosine (5hmC). Various pathways exist to replace the 5hmC with an unmethylated C, thereby bypassing the need to invoke DNA synthesis to eliminate the epigenetic methyl mark. These include amidation of the 5hmC to uracil, or conversion to 5fmC (f = furyl), or 5camC (ca = carboxyl), all of which can be returned to C by BER. Loss of TET3 is lethal to the zygote, attesting to the critical importance of this demethylation event. Other aspects of demethylation unique to this global phase include the following: (1) germ cells are eightfold more methylated in males than in females because of methylation of autosomes, not of sex chromosomes; (2) LINE/LTR retrotransposons are resistant to DNA demethylation during germ cell generation; (3) some maternally imprinted genes in oocytes are also resistant to demethylation; and (4) non-CPG methylation occurs only in male germ cells.[191,254,255] This is an emerging area of research, with surely more surprises to come.

Assisted Reproductive Technologies

An important corollary to both germ cell and zygote epigenetic reprogramming converges with the concept of early life programming. It is becoming apparent that changes to epigenetic profiles can occur as an unintended consequence of ARTs, such as IVF or intracytoplasmic sperm injection. Concern was heightened by reports of increased incidence of imprinting disorders due to improper maintenance of the DNA methylation maintaining the bias in maternal vs paternal expression of a limited but vitally important set of genes. Both Beckwith–Wiedemann[256,257] and Angelman syndrome,[258] which are known but rare imprinting disorders, occur at higher rates in children conceived by ART than in the general population (see Chapter 45). In both cases, the severity of the syndrome is greatest when there are deficits in imprinting of the maternal allele, suggesting the epigenetic reprogramming of the oocyte may be more fragile or more vulnerable to perturbation than that of the sperm.[259] Studies using animal models, both rodents and larger species such as farm animals, have confirmed that there is increased risk of epigenetic alterations associated with aspects of ART, but the precise origins and mechanisms of the risk are unclear, in part because the majority of children born following ART are healthy in contrast to the more consistent deleterious effects observed in animal models.[260] Nonetheless, there is a need for increased awareness, more hypothesis-driven research, and greater transparency in the methods and reagents used in ART in order to assure best practices and reduce risk.[256,259]

Parental Influences on Offspring

Barker and colleagues discovered that infant birth weights were correlated with risk for a variety of adult-onset diseases, including diabetes and hypertension.[6–8] The focus of many studies that assess the negative relationship between low birth weight and adult diseases, including obesity, has been the hypothalamic–pituitary–adrenal axis (HPA) as a source of these metabolic abnormalities.[261] Although Barker was not concerned with behavior or reproduction, his work combined with earlier studies on prenatal stress and sexual differentiation[247] laid the groundwork for a new avenue of research, which helped establish the field of behavioral epigenetics and the notion of early life programming. Much of this work was conducted in Meaney's laboratory at McGill University. Meaney and colleagues were examining the role of stress during the neonatal period on changes in the HPA axis with an emphasis on the permanence of these alterations.[262] The breakthrough came with the discovery that variation in maternal behavior of rat dams changed expression of many behaviors related to stress in adult offspring.[9,263–265] The addition of epigenetics expert Moshe Szyf to the team facilitated understanding of the mechanistic bases for the changes in gene expression, which determined adult behaviors.[144] The idea that maternal care so profoundly affected adult behavior captured many scientists', and the public's, imaginations. The work blended developmental psychology and molecular genetics and has had a transformative impact on the field (Figure 52.17).

Since these discoveries by Barker, Meaney, and others, several paradigms have been used to study the relationship between parental environment and/or behavior and the phenotypes of the adult offspring. Much of this exciting work is at the cutting edge of determining epigenetic mechanisms.

Maternal Licking and Grooming Effects on Male Offspring

In many animals, including humans, there is a high degree of variability in maternal care. One type of care, licking and grooming (LG), is essential in rodents: neonates cannot urinate and defecate without this stimulation. Rat dams display a normal "bell curve" distribution in the expression of these behaviors. When adult offspring from dams on the two extremes of this population are observed, many behavioral differences are noted, including differences in anxiety in males. Differential levels of maternal licking and crouching behavior promote changes in expression of several genes in the stress axis. The first one extensively investigated was the GR, *Nr3c1* in the hippocampus. In an elegant series of studies, the relationship of maternal LG behavior during the critical period (the first week of life) to anxiety in

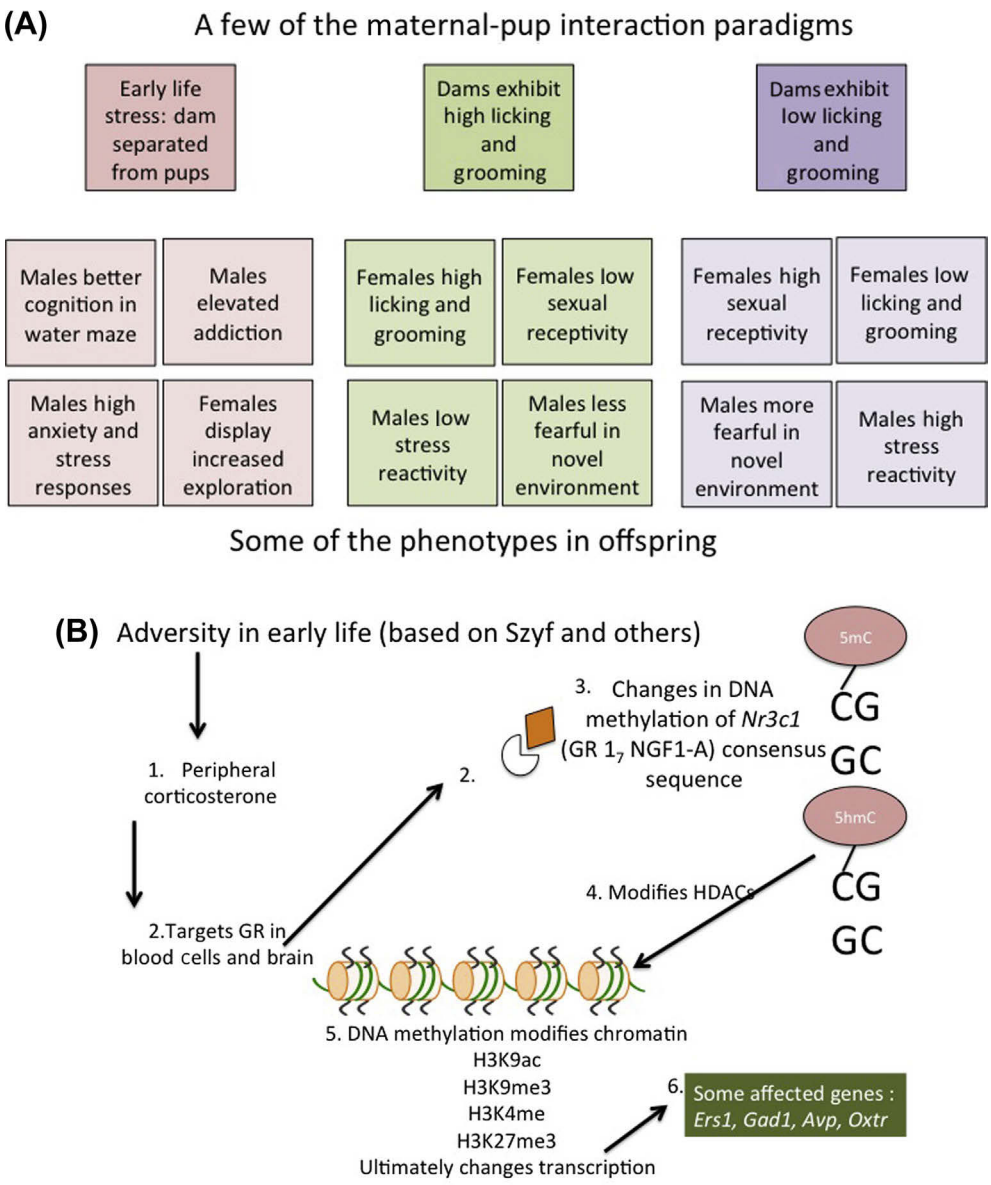


FIGURE 52.17 Maternal behavior impacts brain and behavior. (A) Many paradigms have been used to study how dam-offspring interactions change adult behavior of grown offspring. A sample of behavioral phenotypes associated with early life stress, and natural variation in licking and grooming behaviors are presented. (B) Model for maternal behavioral effects on hormone/epigenetic interactions in pups. Step 1: The behavior directed toward the pup alters the pup's levels of corticosterone. The direction of this change is determined by the amount of maternal care. Step 2: In brain and also in blood cells, the hormone interacts with its receptor. Step 3: Modifications in DNA methylation of Exon 1_7 and NGF1-A consensus site of the GR. Step 4: DNA methylation modifications affect HDAC recruitment. Step 5: HDAC availability affects the amounts and types of chromatin modifications associated with the GR. Step 6: Downstream direct and indirect actions on transcription of other genes. *Nr3c1* = glucocorticosterone receptor, *NGF1-A* = neural growth factor inducible factor A, 5mC = 5-methlycytosine, 5hmC = 5-hydroxymethlycytosine, HDAC = histone deacetyltransferase, *Ers1* = estrogen receptor alpha, *Gad1* = glutamate decarboxylase 1, *Avp* = vasopressin, *Oxtr* = oxytocin receptor.

adult male rats was illustrated (Figure 52.17). The first important aspect of these behaviors is that they are environmentally specified. When pups younger than postnatal day 6 (PN6) from high LG dams are fostered to low LG dams, and vice versa, the behaviors of offspring in adulthood were in accordance with their foster dams, not their biological mothers.[9,266] High maternal LG leads to a decrease in sensitivity to glucocorticoids; thus, as adults, offspring of high LG dams have a reduced stress response.[267] Behavioral differences included decreased startle and burying responses to novel objects, decreased latency to eat, and increased exploration in a novel environment. In addition to *Nr3c1*, high LG offspring had increased mRNA levels of $GABA_A$ and decreased *Crf* in the amygdala.

How could LG of the pups have such a profound effect on their brains and behavior? The initial stimulus for this developmental pathway may be serotonin (5-HT) released in the hippocampus in response to the tactile stimulation of grooming. The serotonin receptor 5-HT7 colocalizes with GR-containing neurons[265]; when 5-HT is present, the protein kinase A pathway is activated and CBP plus another transcriptional activator, nerve growth factor 1A (NGF1-A), bind to the *Nr3c1* promoter.[268] The amount of Ngf1-A at the *Nr3c1* promoter in exon 1_7 is thus differentially expressed: higher in pups that receive more LG stimulation. The percent of DNA methylation on exon 1_7 in turn dictates the amount of *Nr3c1* mRNA. The differences in Ngf1-A were only noted in 6-day-old or younger pups and were not present in the adult brain.[266] This suggested histone acetylation might be required during this critical period to recruit DNMTs to the promoter and change methylation status. To test this hypothesis, ChIP was conducted with hippocampal

tissue using an H3 pan acetylation antibody. More H3 acetylation was associated with the *Nr3c1* exon 1_7 region in pups exposed to low levels of LG as compared to pups receiving high LG. In addition, central infusion of the HDAC inhibitor, trichostatin A (TSA), into brains of pups or diets containing high levels of methyl donors both shifted DNA methylation and resulted in enhanced methylation of the *Nr3c1* promoter.[269] In adults, intracerebral infusion of L-methionine (a methyl donor) reversed the anxiety phenotype in males raised by high LG dams. An exciting aspect of this work is that these epigenetic relationships may also be present in humans. In human suicide victims, a decrease in *NR3C1* mRNA in the hippocampus was associated with increased DNA methylation.[270]

Maternal Behavior and Female Offspring

The same paradigm described above revealed that adult females from low LG dams spend less time on average LG their own pups, and the reverse is true for parturient females from high LG dams.[266] Female reproductive behavior and puberty onset are also affected by maternal care.[271–273] Females that receive low levels of LG as pups attain puberty earlier than females from high LG dams. In paced mating tests, adult females from low LG environments are more receptive to males, receiving more ejaculations (particularly at the beginning of the estrous period) compared with females raised by high LG dams.[274] In group mating tests, adult female offspring from high LG dams received fewer ejaculations initially as compared with offspring of low LG mothers.[275] Over the duration of the test, both types of females received equivalent mating and there were no differences in pregnancy outcomes. Of note, in cross-fostering studies, the offspring of low LG dams displayed receptive behavior like their foster high LG adopted dams. However, the same was not true for the offspring of high LG dams that were fostered. This suggests that a truly genetic mechanism is involved in the lower levels of receptive behavior performed by offspring of high LG dams.

Mechanisms underlying these differences in female reproduction are due to actions on the gene encoding ERα (*Esr1*) and its target genes, such as the receptor for oxytocin.[276,277] High LG dams and their offspring have greater levels of *Esr1* mRNA in the medial POA (MPOA) than do low LG rats. When pups were cross-fostered to the opposite LG type, their *Esr1* mRNA levels in the MPOA corresponded to those of their foster dam. The difference in *Esr1* gene expression can be mapped to methylation of *Esr1* exon 1b, which is greater in the brains of low LG females. One portion of the differentially methylated *Esr1* promoter contains the STAT5 consensus sequence; *Stat5* has been shown to increase expression of *Esr1* via the Jak/Stat signaling pathway. ChIP studies using an antibody to STAT5b have shown that adult female offspring from high LG dams have significantly more STAT associated with *Esr1* promoter as compared with offspring from low LG dams.[276] One gene regulated by ERα that is important for maternal behavior is the oxytocin receptor (*Otxr*). High LG rats have more oxytocin binding in the MPOA than do low LG dams. This is likely due to increased OTR produced by higher levels of *Esr1* in the MPOA of the highly maternal dams. Interestingly, in a different brain region, the amygdala, simulated LG (tactile stimulation) given three times daily prior to 10 days of age enhanced *Esr1* methylation and decreased mRNA in females.[278]

Impaired Maternal Care

One of the more powerful aspects of the studies on maternal LG of pups is that all of the dams behave within the normal range, just at opposite extremes of normal. But what about when maternal care is decidedly abnormal? In other words, can we model parental abuse and neglect in our animal models? Based on the work of Sweatt and colleagues, the answer would be yes. Exploiting the fact that newly parturient rats are distressed by environmental changes, these investigators created an environment in which dams neglected pups, showed little nursing and grooming, frequently stepped on them, and occasionally handled them roughly or dropped them. As a result, there was a dramatic change in the methylation status of the promoter of the *Bdnf* gene, which is implicated in numerous neuropsychiatric disorders but most notably affective disorders, such as depression. Not only was the control of *Bdnf* expression altered in the offspring of abusive dams, it was also altered in their granddaughters (F2), showing multigenerational epigenetic programming. The potential for therapeutic reversal of the deleterious effects of maternal abuse on the epigenome was found in the ability of Zebularine, a DNMT inhibiting enzyme, to reduce the excessive methylation of the BDNF gene promoter and presumably restore normal expression.[279] Unfortunately, behavior was not assessed in this study; however, interestingly, in mice communal nesting increases both maternal care and peer interactions and produces enhanced BDNF protein in the frontal cortex, hypothalamus, and hippocampus.[280] Thus, in addition to pharmacological treatments, social stimulation may modulate BDNF production.

Prenatal (Maternal) Stress Affects Offspring

Prenatal stress has been known for many years to alter behavioral phenotypes in animals, and data suggests it does the same in humans.[281] One classic stress technique in rodents is restraint with simultaneous exposure to a bright light.[282] Offspring of pregnant rats exposed to this paradigm during the last week of gestation have a number of behavioral and neuroendocrine changes. These include changes in sleep/wake cycles, increased

anxiety, decreased learning, and increased immobility in the forced swim test. In general, the male offspring are more vulnerable than the females.[282,283] It is worth noting that several types of enrichments, such as group housing and complex caging with running wheels and objects to explore, have been used to reverse these stress phenotypes.[284,285] One mechanism by which dams' stress is transduced to the embryos is by her glucocorticoids, which are normally inactivated in the placenta by 11β-HSD2. In placenta of chronically stressed embryos, this enzyme is reduced; yet, after an acute stress, 11β-HSD2 is upregulated.[286] Another potential mechanism is via changes in exposure to androgens, which may demasculinize the male central nervous system.[247,287]

An additional source for transducing the impact of gestational stress to the embryos could be the formation of the placenta at the beginning of the pregnancy, which might be susceptible to stress; this could affect nutritional input to the fetus. Indeed, this has been recently demonstrated in hybrid mice. The Bale laboratory has shown that expression of *O*-linked *N*-acetylglucosamine (GlcNAc) transferase (*Ogt*) gene, which is on the X-chromosome, is lower in male than in female placenta. Moreover, when dams experience stress for the first week of their pregnancies, expression of this gene decreases even further in male placenta. These data show profound differences in the sensitivity of males vs females.[288] The role of epigenetics in these processes is beginning to be explored.

Exposing pregnant mice to variable chronic mild stress during the first week of gestation demasculinzines both stress responding in adulthood and brain gene and miRNA expression in the second-generation males. The second generation males from prenatally stressed fathers had a reduced anogenital distance and smaller testis weights compared to offspring of nonprenatally stressed sires, suggesting the behavioral phenotype and stress axis sensitivity may be secondary to reduced testosterone developmentally. However, prenatal stress also reduced brain miRNA levels in four of the seven miRNA assayed in the first generation males and altered the expression of multiple genes relevant to brain development.[289] A shorter period of prenatal stress (gestational days 12–18) consisting of restraint and forced swimming was used to examine the potential role of miRNAs in both maternal and rat pup brains.[290] Frontal cortex and hippocampus from control and stress dams revealed over 300 miRNA that were differentially expressed. In whole pup brains, three miRNAs were enhanced, none of which overlapped with the data set found in mice.

An interesting variation on prenatal stress is "bystander" stress. In this clever paradigm used by Mychasiuk and colleagues, a pregnant rat is housed with another female. To produce indirect stress to the dam and her embryos, the nonpregnant cage-mate is subjected to periodic stress on an elevated platform under a bright light.[291] Exposure to this indirect stress, just during the gestational period, has long-term effects on behavior and brain anatomy of the pups. Young females (9–10 days old) from the pregnant dam subject to bystander stress spent less time pointing upwards on a vertical platform, an indication of disrupted geotaxis, which is an excellent measurement of sensorimotor development. By 3 weeks of age, female offspring of dams exposed to bystander stress weighed less and displayed less locomotor activity in an open field[291] than controls. Histology was conducted to examine the hippocampus and the medial prefrontal cortex. In general, density and dendritic branching in hippocampal neurons are enhanced in 3-week-old male and female pups exposed to bystander stress.[292] Primarily in females, bystander stress also affected numbers of synapses, neurons, and glial cells. Global DNA methylation was higher in the hippocampus and frontal cortex of all rats that experienced this prenatal stress as compared with controls. As expected, gene expression (as assessed by microarray) was depressed in the majority of differentially expressed genes from stressed brains.[291] The array revealed many sex differences as well as effects of stress. One interesting gene that was significantly down regulated in frontal cortex in both sexes exposed to prenatal bystander stress was *Slc6a.1*, which encodes a GABA transporter.

One potential confound with any prenatal stress model is that the dams subjected to stress may provide suboptimal maternal care. Less nursing behavior has been reported in prenatally stressed rat dams as compared with controls.[293] Pups from prenatally stressed dams that were raised by either another stressed or a normal foster rat had normal type 2 GR, dopamine, and glutamate receptors, as determined by receptor binding assays.[294,295] In addition, fostered rats return to basal corticosterone after stress or novelty. This caveat is true for postbirth stress models as well.

Maternal Separation (Early Life Stress)

Yet another way to stress infants and produce alterations in their adult behavior and brains is to remove the dam. Maternal separation is also frequently referred to as early life stress (ELS). ELS paradigms can vary in the length of time the separation lasts, how frequently it occurs, the age of the infants, condition of the infants (if they are left at room temperature or heated), and the distance between the dam and her pups. Neumann and colleagues spearheaded research on ELS using rats and separating pups from dams for 3 h a day for the first 2 weeks of their lives.[296] Males were tested in adulthood and displayed more floating in a forced swim test (an indicator of depression), and higher ACTH and corticosterone after the swim. They also showed more aggression

in a resident intruder test and their *Avp* mRNA and immunoreactivity were higher after the aggressive encounter as compared with normal males. In addition, 5HT immunoreactivity was depressed in the anterior hypothalamus, and this was inversely correlated with aggressive behavior. Using a similar paradigm in mice, ELS increased anxiety in both sexes, decreased attack behavior in the adult male offspring, and decreased maternal aggression in the females. Optical densities of AVP in the paraventricular nucleus (PVN) were elevated by ELS in both sexes, and oxytocin was reduced in females only.[297]

These studies set the stage for more mechanistic work done by Murgatroyd and collaborators.[298] The ELS was conducted with C57BL/6 mice using a 3-h separation for the first 10 days of life. Adult male offspring exhibited many of the same phenotypes as quantified previously.[297] Males exposed to ELS had elevated serum corticosterone, higher *Pomc* mRNA in the PVN, elevated anxiety, and increased motility in the forced swim test. POMC is regulated by CRH and AVP; both genes were examined, and ELS increased *Avp* but not *Crh* mRNA in the PVN. Bisulfite sequencing of CpG sites on the *Avp* enhancer revealed hypomethylation in ELS males between the ages of 6 weeks and 1 year. Interestingly, the number of sites and amount of hypothmethylation declined with age. With a hypothalamic cell line, they went on to show that one particular CpG site was consistently hypomethylated in ELS mice, and this site binds MeCP2 selectively. In vivo, PVN phosphorylated MeCP2 was co-expressed in AVP-containing neurons and in ELS brains. ChIP studies using MeCP2 antibody demonstrated reduced binding to the *Avp* gene promoter. Taken together, the data suggest that the effect of ELS is to increase MeCP2 phosphorylation, thus reducing its binding to the *Avp* gene, which results in decreased methylation and increased gene expression.

This elegant work stands as one of the best examples of the transduction of early life experiences into epigenetic modifications in gene transcripts and changes in behavior. One interesting sidebar is that in C57BL/6 mice, maternal separation for 3 h increased pup retrieval, licking, and/or nursing and nesting while reducing off the nest activity.[299] Thus, in contrast to being raised by a high LG rat dam, mice experiencing ELS show increased anxiety.

Fathers Matter Too

In humans, historical data from Swedish farmers shows that nutritional status of grandfathers (in the late 1800s) had multigenerational effects on a number of metabolic measures in sons and even grandsons.[300,301] These effects can also be seen in mice when the sire experiences a 24 h fast prior to mating. Offspring of both sexes have elevated serum glucose and corticosterone levels.[302] In addition, in mice, dams on high-fat diets prior to and during pregnancy have offspring with reduced insulin sensitivity; when the first-generation males or females are mated, their offspring possess this same trait.[303] In rats, a low-protein diet during gestation produces offspring with hypertension, which can also be transmitted by males or females to the next generation but not to the following (F3) generation, suggesting a multigenerational effect but not a transgenerational effect.[304] The age of the sire can also influence several characteristics; male mice born to very old (120 weeks) sires had lower reproductive success than males with young sires.[305] There are many examples in humans of negative effects of paternal age on various diseases, including autism.[306]

Characteristics of the sire, while less often examined, can also influence the offspring's behavior.[307] In rats, environmental enrichment experienced by the sire for 28 days prior to mating effects both male and female offspring.[308] Counterintuitively, 3-week-old offspring from enriched sires had lighter brain weights, and hippocampus and frontal cortex had less global methylation than found in controls. In males only, offspring (tested on PN10–13 and 15) of enriched sires displayed enhanced activity in an open field. Allowing dams to live in an enriched environment before and during gestation had even greater effects on offspring. Maternal enrichment decreased positive geotaxis in 10-day-old pups and increased activity in the open field in slightly older pups. As was the case for sires from an enriched environment, global DNA methylation was also reduced by enrichment of dams.

Male rats exposed to a brief daily stress on an elevated platform for 27 days prior to mating sired less anxious male pups with a temporary (PN9 but not PN10) impairment in geotaxis shown in both sexes.[309] Global DNA methylation in the hippocampus of both sexes of offspring was increased when sires were stressed. Interestingly, in the prefrontal cortex, no differences were noted in males, while in females stressed sires decreased methylation. It is interesting that prenatal stress to sires vs dams did not have the identical effects on DNA methylation. In the case of maternal prenatal stress, DNA methylation was increased in both the hippocampus and frontal cortical regions.[310] The differences in the frontal cortex may be related to the parental origin of the stress.

When male mice were raised in environmentally and socially enriched conditions, they sired less anxious and heavier pups than males raised in isolation.[311] It is possible that this paradigm may include some bystander effects (see above) because the sires and dams interacted for about 2 weeks—a period that likely encompassed some of the early pregnancy. A study demonstrated a potential contribution of the Y-chromosome to anxiety in female offspring.[312] The authors tend to argue for a

genetic effect, and this may be the case particularly because the PARs of the sex chromosome genes undergo recombination at fertilization. However, the dams and sires were together for at least part of the mating period; thus, the sires' behavior could have had an epigenetic effect on the dams and their pups.

What about Humans?

An exciting aspect of this work is that some of these epigenetic relationships may also be present in humans. The first demonstration of these types of epigenetic changes related to human behavior was work with postmortem suicide victims. In comparison with control brains, suicide victim brains had a decrease in *NR3C1* mRNA in the hippocampus, and this was associated with increased DNA methylation.[270] More recently,[313] brains from suicide victims that did or did not report early abuse were assessed. A specific promoter variant of the *NR3C1* gene was identified in those victims reporting ELS.

To more directly measure the effects of parental stress on offspring gene expression, parents of 15-year-olds rated their stress by self-report for two time periods during the children's early life.[314] DNA samples were collected from more than 100 adolescents. Global methylation was correlated with maternal stress and several genes were also examined for their DNA methylation status in relationship to parental stress, gender of the child, and timing of the stressful period. Questions of causality linger, but taken together these studies demonstrate the potency of early life events, be they adverse or merely variable within the normal range. Epigenetics is poised to transduce environment and experience into semipermanent changes to the brain, thereby optimizing behavioral phenotype in the predicted adult environment.

Transgenerational Epigenetic Inheritance

One of the most exciting—and disturbing—aspects of epigenetics is that it can contribute to inheritance. We typically think about inheritance of genetic traits; however, we now know that different gene alleles, mutations, and copy number variations are not the only way to accomplish inheritance of a behavioral, physical, or reproductive trait in subsequent generations. How epigenetic modifications arise from environmental (nutritional, chemical, or physical) perturbations is the topic of much of this chapter. Inheritance via any of the mechanisms mentioned earlier in the chapter is a whole different aspect that we are just beginning to consider. Before considering the data, we need to introduce a few definitions. As noted previously, in order for the inheritance to be truly transgenerational, the epigenetic (or genetic) changes must manifest in the germ line.[315] To conclude that some trait is going through germline transmission after intrauterine exposure, the trait must be expressed for at least three generations. This is referred to as "germ line" or "transgenerational" inheritance. This three-generation rule is important, as we will explain here.

When a trait is expressed only in the first generation, the terms "social" or "state dependent" are typically used to describe the mode of transmission.[11] One good example is inheritance of maternal style in female rats reared by low vs high LG dams. In each generation, the trait is determined by the pups maternal behavior experience. This is demonstrated by cross-fostering studies in which adult offspring have the maternal style of the dam that reared them, not their biological dam.[316] Thus, the behavioral phenotype is experience dependent.

"Multigenerational" is the best term to describe inheritance in the F1 or the F2 offspring. In several of the cases we have just discussed above, a behavioral or genetic feature was examined in F1 or F2, but it has not been assessed in the F3. One example is the anxiety phenotype in F1 and F2 male mice produced by dams subjected to chronic variable stress for the first 7 days of their pregnancies.[289] This may well be transgenerational, but so far the data only support a multigenerational effect, not genuine epigenetic inheritance. The same descriptions would apply to genetic mutations, which might be caused by exposure of the dam to mutagenic agents that impact embryonic development. In order to be inherited, transgenerational mutations need to be maintained past the F2 generation and into the F3. Importantly, to know if a transgenerational phenotype was genetic or epigenetic in origin, it is necessary to perform DNA sequencing of the essential gene; this would need to include determining which CpG nucleotides were methylated and whether this pattern was faithfully maintained across generations in order to conclude an epigenetic transgenerational effect.

As illustrated in Figure 52.18, when the developing fetus is exposed to an environmental variable (nutritional, chemical, or physical) during gestation, the dam is the F0 generation and the embryos are the F1. There are two periods in development when genome-wide germ cell reprogramming occurs: during fertilization and at the time that germ cells are migrating into the undifferentiated gonad.[252,317] During reprogramming, changes in DNA methylation, histone modification, etc., occur. In the developing F1 embryo, the entire developing body of the fetus, including its germ cells, is exposed to the environmental trigger. Thus, any changes in reproduction or behavior in the F1 may be caused by direct actions of the environment on the developing organism. The F1 animals' offspring are the F2; even at this stage, if the change is maintained, it is not yet clear it is transgenerational because the F2 offspring were F1 germ cells and thus during F1 gestation F2 DNA was exposed directly

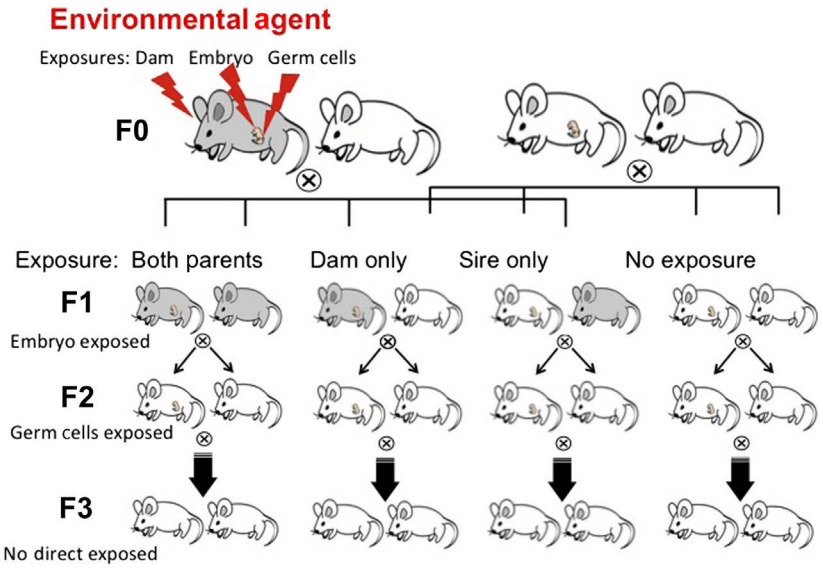


FIGURE 52.18 Transgenerational epigenetic inheritance. To detect transgenerational changes in the epigenome, animals are typically exposed to the environmental agent during pregnancy (F0, gray mouse). Epigenetic remodeling occurs at fertilization and when germ cells migrate to the indifferent gonads; thus, these are optimal times for exposure. The first generation of offspring (F1) will have direct exposure to the environmental agent. These offspring can be mated with similarly exposed or with control partners to create the F2 generation. By pairing exposed dams or sires to control partners, the parent responsible for the inheritance can be determined. The F2 offspring from the exposure line were only exposed to the environmental agent via their germ cells (present in the F1 embryos). Thus, it is likely that phenotypes of F1 and F2 generations will differ. The most important generation for these studies is the F3. If offspring from the exposed vs the control lines have differences in phenotype, they may be due to epigenetic inheritance.

to the environmental trigger. The F3 is the first generation that has had no direct exposure to the environmental trigger. In the case of a postnatal or adult exposure, the F1 germ cells may be affected; thus, the F2 generation needs to be assessed.

One of the other interesting aspects of F1, F2, and F3 (or greater) inheritance is that the phenotype may be different in different generations. For example, the actions of a prenatal exposure to the endocrine disrupting compound (EDC) bisphenol A on F1 behavior may be the opposite of the effects in F2 or F3, and this may be explained by how the compound acts on the developing brain vs the germ cells. We will describe an example below. Another aspect of transgenerational inheritance that we will discuss is the issue of the parent of origin. One dichotomy between the sexes is that male germ cells continue to go through mitosis throughout the lifespan. Female germ cells have partly completed mitosis by birth; within each cycle after puberty a subset of these, the ones destined to ovulate, complete the process as part of preparation for fertilization.

Endocrine Disrupting Compounds

Studies of EDC effects on reproduction were stimulated by two chemicals in particular: 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) and diethylstilbestrol (DES). DDT was developed to help control lice in soldiers during World War II and afterward was widely used as an insecticide. It was banned in the early 1970s in the US after the negative impact of this compound on wildlife was revealed. There is a clear link between breast cancer and prepubertal exposure to DDT.[318] There are also F1 effects of paternal exposure to DDT on other cancers[319–321] and hypertension.[322] DDT can bind to ERs and ARs; it is lipophilic and breaks down very slowly, persisting in the environment long after its use has been discontinued.[323] Despite the ban, DDT is still present in detectable levels in blood samples of pregnant women in Texas and Peru, to name a few locations.[324,325] Studies are underway to determine if F2 effects are evident.

DES was prescribed to pregnant women at risk of miscarriages. The idea was that this treatment would maintain pregnancies; however, it failed to do so and instead produced abnormal reproductive effects. Daughters of women given DES during their pregnancies tended to have more reproductive abnormalities than unexposed women.[326] Dose-response studies in mice showed that doses as low as 0.002 μg/pup resulted in 30% incidence of uterine tumors in adults, and these studies provided evidence of an inverted-U dose–response curve for these effects.[327] Inheritance of these effects in offspring of both sexes has been demonstrated (up to the second generation) in mice[328] and confirmed by recent human data. In a population study of DES-exposed families, sons of DES-exposed mothers had a small, but significant, increase in hypospadias (3.57% as compared to none in unexposed controls). In the next generation (grandsons of DES-exposed mothers), the incidence increased again to 8.2% and none of the control grandsons had hypospadias.[329] The next generation of results will be critical to see if this effect has gone into the germ line. These examples illustrate that the parent of origin (the mother) does not have to be the same sex as the affected offspring.

Not surprisingly, manmade EDCs have received the largest amount of attention regarding their potential transgenerational effects.[330] Starting in 2005, Skinner and colleagues reported transgenerational actions of the antifungal agricultural agent, vinclozolin, and the pesticide, methoxychlor, on fertility in male rats.[331] Pregnant rat dams from gestational days 8–15 (during germ cell

migration) were treated with one of the EDCs, or control injections and the subsequent generations of male offspring were monitored. Starting in the first generation and continuing through F4 males, ancestral lines who had received vinclozolin or methoxychlor had increased cell death in the testes, fewer sperm, and reduced sperm motility. Using outcrossing, they determined that the parent of origin for this effect was the sire. Subsequently, studies focusing on vinclozolin have reported transgenerational incidence of increased tumors; diseased-appearing tissues in kidney, prostate, and testes; and inflammation in many regions.[332] In females, uterine abnormalities that interfered with pregnancy were noted along with increased glomerular abnormalities in kidneys.[333] This work has been extended to behavior. Males in the F3 vinclozolin lineage are less anxious (and/or more active) than controls, while the opposite effect is seen in female rats.[334] Of note, vinclozolin-exposed males (F3) are less attractive than control males when females were tested for partner preferences.[335]

Given the abnormal cell growth produced by vinclozolin, it is important to know whether its actions are directly on DNA (gene mutations) or epigenetic. Skinner has argued that the majority of EDCs, particularly in the doses most humans are exposed to, do not cause DNA mutations, but they can affect the epigenome. He rationalizes that the high frequencies of phenotypes is incompatible with the random nature of DNA mutations, which would affect less than 1% of all genes.[336] Vinclozolin toxicology studies have been conducted in mice, human cells, yeast, bacteria, and cell lines.[337,338] Using relatively high starting concentrations (5 μM or 1 μg/culture or 625 mg/kg body weight in mice), some studies report changes in various indirect markers of genetic mutations, such as chromosomal abnormalities, sister chromosome exchange, cell nuclei numbers, and activity of carcinogenic metabolic enzymes. However, in light of the high concentrations used, the lack of inheritance data, and use of indirect markers, it is not likely that the doses used in vivo by Skinner (e.g., 100 mg/kg body weight) and others are producing DNA mutations.

The Skinner laboratory has examined the impact of a number of other EDCs (in mixtures) in F3 offspring, recently.[339] The combination of phthalates and bisphenol A, at two doses, several dioxins, and jet fuels all accelerated puberty onset in female rats; all these chemicals, plus a mixture of pesticides, reduced the number of follicles in the ovaries. In F3 males, serum testosterone was reduced significantly by all the EDCs except the pesticides. In addition, the effects of vinclozolin exposure in utero on F1 through F3 males and females were assessed in two mouse strains.[340] Sperm cell apoptosis was increased transgenerationally in mice along with disease phenotypes in kidney, prostate, and testes. In females, the numbers of adults with ovarian cysts increased with generation.

Bisphenol A (BPA) has transgenerational actions on fertility in rats. Dams were treated during pregnancy with one of two doses; in subsequent generations, their daughters were mated and resorbed fetuses were noted. In F1, F2, and F3 offspring, reabsorption rates were higher in BPA vs control lineages.[341] In agreement with this observation, litter sizes were reduced. In male offspring, body, epididymis, and prostate weights were increased by BPA exposure in F2 and F3 generations. Sperm counts and motility were also reduced. In rat testes, immunocytochemistry was used to quantify steroid receptors and several co-activators.[341,342] AR, ERβ, SRC1, and NCor, were all lower in BPA males across all generations. BPA also acts on the brain in a transgenerational manner. In mice, dams were fed a low dose of BPA in chow prior to and during pregnancy. Juvenile males and female offspring displayed decreased social interactions in the F1, but more interactions in F2 and F4 generations.[164] In a social recognition test, F1 mice exposed to control diet or low-dose BPA chow in utero displayed normal habituation when the same female was presented over time and dishabituation when a novel female was presented. However, in the F3 mice, the BPA lineage did not dishabituate in this task; yet, olfactory discrimination was normal.[343] This suggests a specific social impairment. In embryos collected at E18.5, whole brains from the F1 and F3 generations had lower expression of *Avp* in F1 and F3. In F3 males, only *Oxt* mRNA was also depressed by BPA.[164]

The effects of gestational exposure to phthalate di-2-ethylhexyl (DEHP) have been examined transgenerationally in mice.[344] Females were fed by gavage once a day during the second week of life with control or DEHP-containing corn oil. Puberty was delayed in all generations (F1–F3) of males, and F3 males had reduced sperm counts, testicular germ cell function, and increased incidence of abnormal seminiferous tubules. These phenotypes were noted in F3 males regardless of the parent of origin. Polychlorinated biphenyls (PCBs) were banned in the 1970s but still persist in the environment and in fatty tissues. Mice received one of four doses in their chow starting on the day of mating, and continuing throughout lactation.[345] Ovary and testes weights were reduced in F1 mice by PCBs in all generations, and males had reduced anogenital distances, but none of these effects were transgenerational. Dioxins (TCDD) are another class of EDCs that are found throughout the world. In mice, exposure of dams to TCDD during pregnancy reduced the number of full-term deliveries in F1 and F3 daughters.[346]

The issue of dose and its relevance to human exposure is central to all studies of EDCs. The work on BPA by Wolstenholme[164] used low doses quantified by high-performance liquid chromatography (HPLC) measurements of blood from treated and untreated mice. The

studies by Salain[341,342] used somewhat higher doses, but no quantification was performed, so it is not clear how relevant these BPA treatments were for humans. The study by Pocar[345] and colleagues was designed to mimic levels of PCB found in breast milk. All the other studies used higher doses that are not likely to be representative of those experienced by most people. It is possible some of these compounds cause DNA mutations at these high doses. Using a comet assay to assess DNA damage in sperm, a study found more damage in males consuming higher than human-relevant doses of BPA as compared with lower dose or no BPA.[347] The group also assessed the "dominant lethal mutation rate" based on calculations of the success in mating, pregnancies, corpora lutea implantations, and live fetuses at E15. A mutation rate of 10% was calculated in males exposed to 4 weeks of daily oral intake.

Another issue to consider is the method of delivery. Most EDCs are ingested but some are inhaled. A few of the studies mentioned used one of those methods, as appropriate. The work done in the Skinner laboratory only used injections, typically intraperitoneal.[334] Many other studies have treated mice and rats orally with EDCs via gavage, which may cause stress that could interact with the actions of the EDCs.[348] A final consideration is the timing of the EDC exposure. There are two prenatal periods when reprogramming occurs; all of the studies cited above except one specifically targeted the midpregnancy period, when PGCs are migrating into the gonads. Wolstenholme placed mice on the BPA-containing diet 10–14 days before pairing and the mice were maintained on those diets for the entire pregnancy; thus, both organizational periods were exposed.[164]

Finally, an additional aspect of EDCs is that they appear to affect gene imprinting status. Over two generations, vinclozolin exposure during pregnancy had a long term (F3) effect on methylation of *Peg3* in sperm.[349] BPA can also disrupt genomic imprinting.[349,350] Using two doses of BPA, both embryonic (E9.5 and E12.5) and placental biallelic expression of several imprinted genes increased. This was accompanied by a reduction in global methylation. Expression of these genes was either enhanced or reduced compared with controls, particularly when the embryos were exposed to the higher of the two BPA doses. Interestingly, this paper reported greater effects of BPA than an earlier study.[351] The major difference was that in the earlier study embryos were only exposed to BPA during germ cell migration, whereas Susiarjo and colleagues fed mice BPA for 2 weeks prior to mating, thus encompassing the fertilization period. If imprinting is influenced by EDCs, it is possible that X-inactivation might also be dysregulated. A summary of environmental toxins demonstrated to have transgenerational effects can be found in Table 52.3.

Paternal Stress

In a series of intriguing studies, the Mansuy laboratory has shown that the combination of stress during pregnancy and maternal separation postpartum creates antisocial behavior in male but not female mice.[356] In a juvenile social encounter, the F1 males from the stress background are not less investigatory of a novel conspecific as the unstressed controls. However, F2 and F3 males from the stressed background were significantly less investigatory than controls. Interestingly, the males produce sons with the same phenotype. Importantly, this effect can be seen in males two generations past the initial stress,[282] making it truly transgenerational. Interestingly, the females that mated with the socially isolated sires had a positive correlation between their nursing frequencies and expression of BDNF mRNA; the opposite relationship was noted for MeCP2 expression.

Potential Mechanisms for Transgenerational Inheritance

In general, epigenetic modifications in gametes are cleared and then reset during two stages in development: fertilization (preimplantation) and during migration of the PGCs to the undifferentiated gonads.[357] However, as is clear from the data presented above, some modifications persist, and these can affect transgenerational changes in gene expression. Details on which mechanisms are involved differ in different species. Zebrafish display asymmetry in remodeling of DNA methylation during these time periods. Only the maternal methylome is remodeled, while the paternal methylome is stably inherited.[358,359] In addition, histones in zebrafish sperm are not associated with protamines and thus are good targets for epigenetic inheritance. In *Drosophila* and *C. elegans*, the chromatin modification H3K9me2/3 and piRNA have been implicated in epigenetic inheritance. Several other chromatin modifications are also important in *C. elegans*.[360] Mutants for the worm homolog for the histone demethylase 1A gene (LSD1/KDM1), which demethylates H3K4me2, have smaller brood sizes; this trend increases over generations.[361] In these animals, the H3K4me2 mark increases in germ cells, leading to sterility over generations.

In mice, most of the work on transgenerational epigenetic inheritance has focused on DNA methylation. This bias is probably due to the long-held view that this epigenetic modification is permanent, although we now know this is not the case.[362] Moreover, during reprogramming in sperm, the protomines were thought to completely replace histones, but we now know this process is not complete, with 5–15% of histones remaining.[363] In known fertile patients, histone retention is found at the promoters of genes important in the embryo, including

TABLE 52.3 Endocrine Disrupting Compounds and Toxins that Impact Epigenetics

EDC	Common Sources	Species	Target for Transgenerational or Epigenetic Action	References
Vinclozolin	Fungicides	Rat (Sprague-Dawley)	Sperm	331
		Rat (Sprague-Dawley)	Tumors in many organs	332
		Rat (Sprague-Dawley)	Immune cells, kidney abnormalities	333
		Mice (FVB/N)	Imprinted genes, sperm	349
		Rat (Sprague-Dawley)	Social behavior, stress reactivity, anxiety	248,334,335
Bisphenol A	Plastics, thermal receipts	Rats (Holtzman)	Sperm, litter size, body weights, epididymal weights, AR and ERβ in testes	341
		Rats (Holtzman)	Intensity of SRC-1, NCor, GRIP, p300/CBP in testes	342
		Mice (C57BL/6J)	Imprinted genes brain expression Oxt and Avp, social behavior	164
		Mice (C57BL/6J)	Imprinted genes	350
Di-(2-ethylhexyl) phthalate	Flexible plastics	Mice (CD1)	Puberty (males), sperm, epididymis, testes	344
		Mice (CD1)	Sperm, gonads (male and female)	340
Polychlorinated biphenyls	Lubricants, paper, eclectic transformer	Mice (CD1)	Testes, sperm	345
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	Herbicides	Mice (C57BL/6)	Pregnancy-to-term birth	346
		Rat (Sprague-Dawley)	Tumors in various organs both sexes	339
		Mice (FVB/N)	Imprinted genes	352
Benzo(a)pyrene	Coal	Mice (C57BL/6)	Sperm	353
Hydrocarbon mixture	Jet fuels	Rat (Sprague-Dawley)	Ovary, body weight	354
Plastics mixture	Plastics	Rat (Sprague-Dawley)	Puberty (female), tumors in various organs both sexes	355
Permethrin and *N*,*N*-diethyl-meta-toluamide mixture	Pesticide and insect repellent	Rat (Sprague-Dawley)	Puberty (both sexes), tumors in various organs both sexes	339

developmental gene promoters, miRNA clusters, and imprinted loci, suggesting that the nucleosome retention is programmatic in nature.[364] In sperm, genes enriched at H3K27me3 are repressed in the early embryo; thus, this modification may contribute to male transmission of epigenetic inheritance. Another source of evidence implicating histones is that the HDAC inhibitor, TSA, given to male mice resulted in a dose-dependent decrease in spermatogenesis and testis weight as well as a decrease in histone deacetylase activity,[365,366] More studies on the role of histone modifications in mammalian epigenetic inheritance are needed.

Much of the epigenetic inheritance data come from work with a variety of EDCs.[164,331,342,344,345] However, only Skinner and colleagues have examined mechanisms. They found that the parent responsible for the transmission of a number of phenotypes is the male and have focused on promoter-wide DNA methylation changes in sperm in F3 lineages exposed originally to vinclozolin vs control in both rats and mice.[331,332] Their techniques include methylation-dependent immunoprecipitation (MeDip) followed by DNA tiling arrays and validation with real-time polymerase chain reaction (PCR). Differentially methylated regions (DMRs) have been noted. Some of the 16 DMRs contain known genes, and one association could be attributed to copy number variations.[340] The genes of interest typically have a low number of CpG repeats. Skinner and

colleagues propose that DMRs in both mouse and rat sperm are similar to imprinting sites. These sites protect the DNA from reprogramming during the epigenetic remodeling periods in development. Using bioinformatics programs, they further suggest several differential methylation motifs (EDMs) that are present within DMR and similar in mice and rats. Direct tests of these ideas will be insightful.

Hypothalamic Control of the Pituitary–Gonadal Axis

At some point in postnatal development in the majority of mammalian females, the surge-release of gonadotropin releasing hormone (GnRH1, subsequently referred to as GnRH) produced in a discrete population of hypothalamic neurons is coordinated with the other aspects of hypothalamic–pituitary–gonadal axis function (see Chapters 26–28 and 30–32). The first time this happens, females move from reproductive immaturity to fertility. Puberty is marked by changes to the neural control of luteinizing hormone (LH) and follicle stimulating hormone (FSH) release, as well as by appropriate changes in behavior, which must be achieved for reproductive success. The neural integration of environmental inputs that affect the timing of puberty and the maintenance of reproductive competence in adults have been recognized for decades. Nutrition, proximity of mates, photoperiod, temperature, and a host of other signals indicate the feasibility of successful reproduction; not surprisingly, all of these modifiers can have epigenetic effects.

GnRH is produced in a small number of neurons that reside in the hypothalamus and project to the median eminence (Figure 52.19) (see Chapter 11). Steroid hormones do not directly regulate the activity of GnRH neurons. However, cell populations in the arcuate nucleus (Arc) and in the anteroventral periventricular nucleus (AVPV) express ERα and AR and are regulated by estrogen and androgen; these cells produce kisspeptin (KISS1).[367] KISS1 can directly regulate GnRH neurons; this neuropeptide and its gene, *KISS1*, are essential for normal puberty.[368] In addition, these cells produce neurokinin B and dynorphin, leading to them being referred to as KNDy neurons in some instances. Other components of the hypothalamic network are substances produced by glia, particularly growth factors. The genes encoding these factors, and others, comprise the GnRH-regulation gene network.[369–371] Thus, all these genes are potential targets of epigenetic modulation.

In rodents, the two main populations of *Kiss1* neurons respond differently to estradiol. In the Arc, estradiol down regulates *Kiss1*, while in the AVPV the steroid has the opposite effect; it is this population of *Kiss1* neurons that controls the ovulatory GnRH surge in these species. Each anatomical region contains a different splice variant of the *Kiss1* gene,[372] and histone modifications play a role in the regulation of the *Kiss1* gene. In a non-*Kiss1* expressing immortalized hypothalamic cell line (N6), treatment with the HDAC inhibitor TSA increased expression of *Kiss1* mRNA.[372] In contrast, the DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC), had no effect on expression of the *Kiss1* gene in this cell line. Using a luciferase construct, a transgenic mouse with

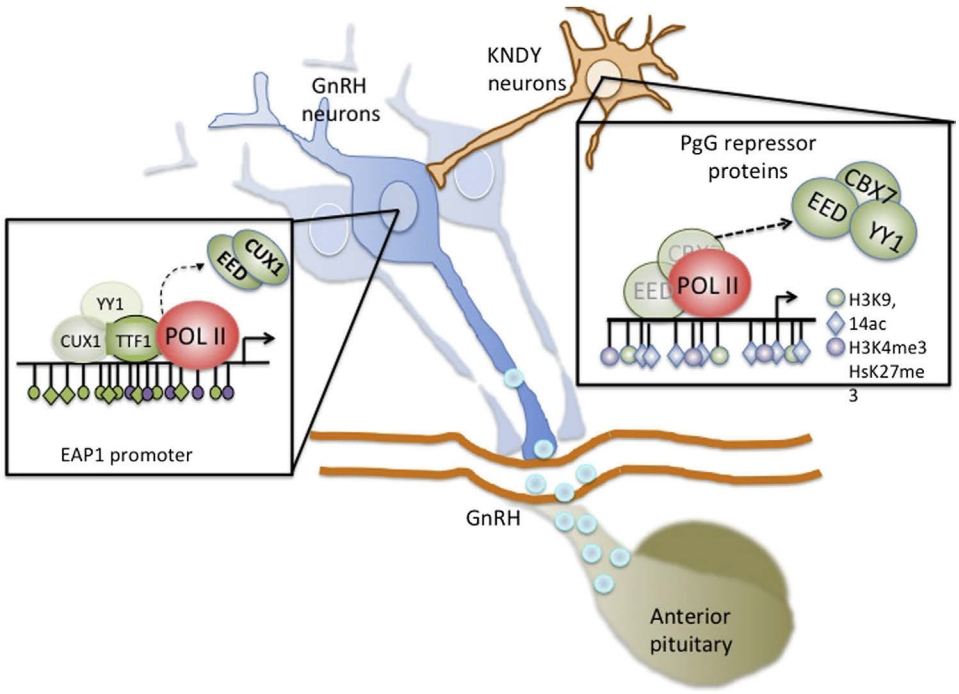


FIGURE 52.19 Epigenetic regulation of GnRH neurons. GnRH release from neurons is regulated in part by kisspeptin (KISS1) produced in KNDy neurons. Expression of the *Kiss1* gene, however, is repressed by polycomb group proteins (EED, CBX7, YY1), as illustrated in the righthand box. Permissive changes to the histones associated with the *Kiss1* promoter release the transcriptionally repressive proteins and allow transcription by RNA polymerase II (Pol II) to proceed. Several of these polycomb group proteins also block *Eap1* (enhanced at puberty 1, left hand box) in GnRH neurons (and perhaps in KISS1 neurons as well). When they are removed from the promoter, GnRH release may proceed.

labeled *Kiss1* neurons was produced and ChIP allowed for examination of hyperacetylation in the two populations of *Kiss1* neurons. Ovariectomized (OVX) mice treated with estradiol, as compared with untreated OVX mice, had higher H3 acetylation in the *Kiss1* promoter from AVPV neurons. This finding was replicated in a comparison of naturally cycling diestrous vs proestrous animals. In contrast, the reverse results were noted in cells from the Arc, suggesting that estradiol acetylated the *Kiss1* promoter in the AVPV but deacetylated the promoter in the Arc.[372] Chromatin confirmation capture performed on AVPV cells showed that estradiol enhances a chromatin loop between the *Kiss1* promoter and the 3′ intergenic region. In the Arc, the 5′ and 3′ regions of the gene were associated with the promoter regardless of the estrogen status of the mouse. Finally, DNA methylation of the *Kiss1* promoter did not differ in either of the subpopulations of kisspeptin neurons, nor in N6 cells and the *Kiss1* expressing cell line, N7.[372]

The expression of at least two PcG genes, embryonic ectoderm development (*Eed*) and chromobox homology 7, decrease prior to the onset of puberty as a result of increased methylation in their promoters, and EED is dislodged from the *Kiss1* promoter (Figure 52.19). This in turn leads to coordinated changes in H3 of the *Kiss1* chromatin that are conducive to gene activation, thereby providing a means for increasing KISS1 protein without changes to the methylation status of the gene.[373]

In contrast to the *Kiss1* population in the Arc, DNA methylation may be important for the sex difference in the AVPV *Kiss1* neurons.[374] Females have more neurons expressing *Kiss1* in this region than do adult males. This sex difference is caused by the actions of early postnatal estrogens derived from aromatization of androgen, which typically only males experience as a product of the normal developmental surge in testicular testosterone (see Chapter 47). When the HDAC inhibitor valproic acid (VPA) was given for 2 days after birth, it reversed the sex difference in the size of the bed nucleus of the stria terminalis (BNSTp).[375] However, this treatment had no effect on the *Kiss1* sex difference. In addition, bisulfite sequencing followed by pyrosequencing of the *Kiss1* gene in the AVPV revealed small differences in percent methylation in four of the seven CpG islands assessed. In all cases, the gene was less methylated in males than females. This finding is counterintuitive and may be caused by a dilution effect because the assessed brain regions contained more than *Kiss1* expressing cells. Alternatively, other sites in the *Kiss1* gene may have sex differences in methylation in the opposite direction. Finally, lower methylation levels in males might allow for more binding of transcriptional repressors—several of which are present on the *Kiss1* gene.[376] In any event, this provides an interesting example of a case where the relationship between DNA methylation and gene expression is not always as predicted.

One recently discovered gene in the GnRH-regulation network is enhanced at puberty (*EAP1*). In a series of studies, Ojeda and colleagues compared RNA and DNA expression profiles from nonhuman primate females selected at a number of times relative to puberty.[377] They also collected human hypothalamic hamartomas associated with early pubertal onset. Bioinformatic analyses revealed a number of genes common to tumors and expressed in postpubertal brains. *Eap1* is a gene encoding for a transcription factor that activates the promoter of the GnRH (*Gnrh1*) gene in neuronal and nonneuronal cell lines.[378] Interestingly, in cell lines, *Eap1* is activated by thyroid transcription factor 1 (*Ttf1*) and repressed by cut-like homeobox (*Cux1*) and yan yang 1 (part of the PcG complex). Both CUX1 and TTF1 are associated with the *Eap1* promoter in pubertal female rat brain.

Several studies support the role of epigenetic modification for expression of *Gnrh1*. The immortalized GnRH cell line GT1-7 displays active histone marks H3ac and H3K4me3, whereas immature GnRH cells (GNII) and non-GnRH cells do not.[379] Two HDAC inhibitors, TSA and VPA, can decrease GnRH expression in GT1-7 cells.[380] This is likely accomplished by repressing *Otx2*, a homolog of the *Drosophila* orthodenticle homeobox 2. When *Otx2* is deleted in mice, this reduces GnRH neuron number, delays puberty, and produces infertility.[381] In parallel with changes to *Gnrh1* gene-associated chromatin, analyses of methylation status of cultured primate GnRH neurons derived from the nasal placode of fetal monkeys revealed a developmental decrease in methylation of the promoter, which parallels increases in intracellular calcium signaling and spontaneous pulsatile GnRH release—clear indicators of maturation.[382]

Mechanisms that regulate puberty are not likely to be restricted to DNA methylation and chromatin modifications. For instance, the onset of vaginal opening in mice was influenced by a LINE element.[383] Thus, there appears to be coordinated epigenetic changes occurring in multiple components of the neural axis governing puberty. A cohesive picture of all the coordinated changes seems nearly within our grasp. However, given how complex and important this function is for evolution, it is likely to be regulated not only by many genes, but by multiple levels of epigenetic modification of these gene players.[384]

Epigenetics and Sexual Differentiation of the Brain and Behavior

The culmination of successful puberty is the production or final maturation of gametes delivered to the reproductive tract, combined with the appropriate motivation to seek out and mate with high-fitness individuals of the opposite sex. Both the strategies and choices regarding

a mate vary between males and females as a function of investment in the offspring. In most mammals, the energetic investment in each embryo is usually large and the female is likely to be the major if not sole provider of food and shelter postpartum. Thus, her reproductive decision making may involve dominance status of the male. In most bird species, the parental investment after egg-laying can be more equitable; thus, mate choice may be more influenced by variables related to territory size, courtship displays, resource provision, etc. In either situation, the brain must be in synch with the gonads so that mating occurs when the female is physiologically ready for insemination and fertilization. To assure this, the brain is preprogrammed developmentally so that an animal possessing male gonads, or testes, will exhibit male reproductive behavior as an adult, while an animal possessing functional ovaries will exhibit female reproductive behavior and all its attendant nuances. This preprogramming is a process called sexual differentiation of the brain[385] (Figure 52.20), to distinguish it from both sex determination (i.e., testes vs ovaries) and sexual differentiation of the secondary sex characteristics. Differentiation of the brain is a direct by-product of sex determination in that a developing male produces large amounts of androgen from the embryonic and neonatal testis, whereas the female ovary is largely quiescent. The gonadal androgens gain access to the brain where they, or estradiol, "differentiate" the male from the female by directing neural development that will favor expression of male sexual behaviors in response to postpubertal androgens and appropriate external stimuli, such as the sights, sounds, and smells of females (see Chapter 47).

A hallmark of this early preprogramming, or sexual differentiation of the brain, is that it occurs during a restricted sensitive period. In the laboratory rat and mouse, this period is the last few days of gestation and the first few days of life. The sensitive period is operationally defined by the onset of gonadal hormonal production in the male and the offset of responsiveness of the female to exogenous hormone administration. Thus, a gonad-intact female can be programmed to have a "male" brain by administering a masculinizing dose of testosterone to her during this perinatal sensitive period. Moreover, in many animals, some of the actions of testosterone on the developing brain are in fact mediated by its aromatized end-product, estradiol, and so administering large doses of estradiol to neonatal females is just as effective at masculinizing many end points as is testosterone. The need for large doses stems from the fact that circulating alpha-fetoprotein binds estrogens in the bloodstream that originated with the dam, so that inadvertent masculinization of female fetuses is avoided. Administrating large doses of estradiol exceeds the binding capacity of the alpha-fetoprotein and allows access to the brain.[386,387]

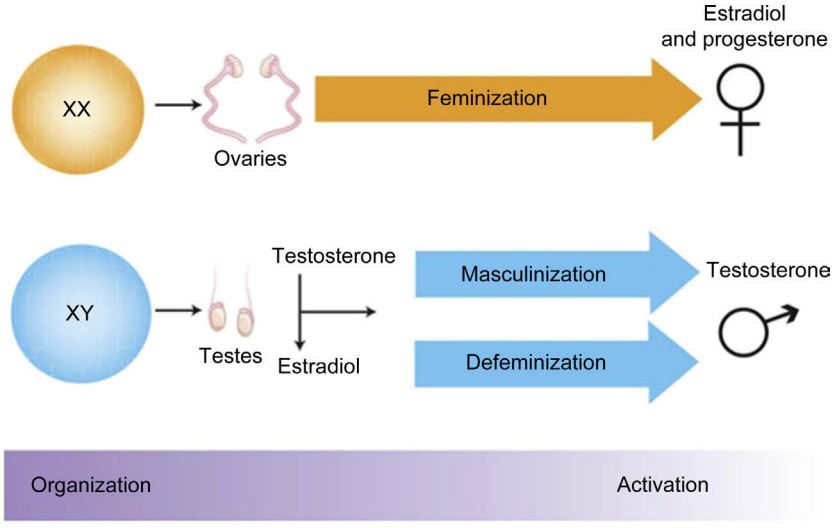


FIGURE 52.20 Sexual differentiation of the brain. Sexual differentiation of the brain begins with the chromosomal complement, which determines gonadal sex. Testosterone synthesized and released by the fetal and neonatal testis can be aromatized to estradiol and both steroid hormones act on the developing brain to both masculinize and defeminize it in males. The female brain develops in the absence of a high level of gonadal hormones during this early sensitive period. Hormone action at this time is referred to as organizational because it sets the stage for adult activational effects, which induce stereotypic behaviors in males and females in response to gonadal steroid production. The organizational effects of steroids are believed to endure at least in part due to epigenetic changes induced during the sensitive period, but there is also evidence for new hormone-dependent epigenetic changes in the adult brain. For the past 50 years, the prevailing view of sexual differentiation of the brain has been a linear model in which chromosomal sex determines gonadal sex, which determines brain sex. This iconic model based on the organizational/activational hypothesis has proved a sturdy framework for elucidating some, but not all, of the aspects of sexual differentiation of the brain. *Source: Reprinted from Ref. 385.*

The actions of gonadal steroids, androgens, and estrogens on the developing brain are termed "organizational" as they are complimented by adult activational effects, such that the hormonal milieu of the adult will now induce the appropriate physiological and behavioral responses. Some are suggesting that this is really more akin to early life programming, only the agent of change is steroids instead of diet or adversity (see Chapter 47). More specifically, in rats, the presence of elevated testosterone in a masculinized brain will regulate pulsatile release of LH from the anterior pituitary with no LH surge, and it will promote male sexual behavior (i.e., mounting the female, intromission, and ejaculation, if capable). Conversely, a feminized brain, in addition to regulating pulsatile release of LH, will respond to sequential estrogen and progesterone exposure with cyclical LH release that includes an ovulation-inducing surge and female sexual behavior (i.e., proceptive solicitation behavior and lordosis in response to appropriate somatosensory stimuli from the male). In both cases, if the adult hormonal milieu does not match that of the developmental exposure, there is a failure of reproductive competence, both behaviorally and physiologically. The requirement for convergence in developmental and adult hormone action is a central tenet of reproductive neuroendocrinology and is referred to as the organizational/activational hypothesis, following an iconic paper in 1959 by Phoenix, Goy, Gerall, and Young.[388]

A critical aspect of the activational component of this two-phase process is that a memory or imprint of the previous organizational component must be accurately and reliably maintained. For many years, this was thought to be accomplished via permanent morphological or structural changes so that differentiated end points such as cell number, axonal projections, and synaptic patterns would be established and then be essentially immutable—a view reflected in our vernacular with the use of words such as the organizational "blueprint" and the "wiring" of the neonatal brain. Although some aspects of this view are no doubt true, we have since gained a greater understanding of the brain's capacity for change and a realization that synapses come and go, cell genesis and cell death are continuous processes even into adulthood (albeit restricted), and that even the strength of axonal projections can change.

Given this high degree of plasticity, an obvious question is how can the earlier hormonally mediated organizational effects be maintained until adulthood when there is an extended intervening period that is largely void of any circulating gonadal steroids? Epigenetics is the likely answer, and indeed steroids are perfectly poised to exert epigenetic influences given that the receptors are nuclear transcription factors that directly interact with the DNA and associate with myriad nuclear proteins, including those with HAT activity, such as Src1 and CBP/p300. Moreover, steroid receptors themselves appear subject to epigenetic regulation as well as indirectly exerting epigenetic influences by modulating key enzymes and other regulatory elements. Although still in the very early phases of discovery, sex differences in brain epigenetics are emerging as key regulators for both establishing and maintaining functional divergence, and perhaps convergence, in males and females.[123,389–391]

Two approaches can be used to explore epigenetics and brain sexual differentiation: (1) determine if there are sex differences in epigenetic parameters in brain regions known to be different in males and females and relevant to reproductive end points and (2) disrupt mechanisms by which epigenetic marks are established and determine if this impacts on the organizational effects of steroids and thereby interferes with or dampens the activational effects. Toward the first approach, sex differences in both histone modifications and DNA methylation of steroid receptors have been identified.

Chromatin Remodeling and Brain Sexual Differentiation

Remembering that hyperacetylated histones H3 and H4 are associated with activated gene transcription while deacetylation results in repression, the levels of these have been measured in various brain regions, including those known to be subject to sexual differentiation. The POA is a key brain region for the expression and regulation of male sexual behavior (see Chapter 49). The activational histone marks H3K9/14ac and H3K9me3 were examined in mouse embryos and neonatal pups; contrary to expectations, there were no sex differences observed in the hypothalamus/POA. However, there were sex differences in the cortex and hippocampus; while the higher levels of H3K9/14ac in males could be attributed to gonadal hormones, the higher levels of H3K9me3 could not, suggesting a role for sex chromosome complement in establishing this particular sex difference.[392] This approach reveals interesting sex differences in epigenetic marks in the brain, but to date does not tell us the functional significance of the differences.

Using the alternative approach of first identifying a sex difference and then determining if there is an epigenetic underpinning to it, Forger and colleagues focused on one sexually dimorphic nucleus, the principle subdivision of the BNSTp. This brain region is characterized by a sex difference in apoptosis, such that males and females start with the same number of neurons but many of them undergo preprogrammed cell death in females, but not in males. Administration of male hormones to females prior to the onset of the critical period for cell death rescues the neurons from dying in females. However, there is a lengthy delay between hormone action and the cell death, leading to the question of whether

steroid-mediated epigenetic programming of the chromatin was involved. Forger and colleagues tested this hypothesis via broadband pharmacological inhibition of HDACs using VPA, which increased H3 acetylation in the BNSTp. Blocking brain HDAC activity in newborn male mice or females treated with a masculinizing dose of testosterone also prevented the neuroprotective effects of both the endogenous (males) and exogenous (females) hormones, resulting in a mature BNSTp that was the same size as that seen in unmanipulated females (i.e., smaller).[375] This is the first demonstration of hormonally mediated epigenetic programming of a structural change in the brain, and it includes changes to vasopressin innervation, not all of which involve the BNSTp. A lack of any effect of VPA treatment on two other brain nuclei speaks to a degree of specificity in the effect, but the genes affected by the chromatin remodeling in males vs females have yet to be identified.

In a combination of the two approaches, Matsuda and colleagues identified candidate genes for epigenetic analyses and used a pharmacological approach to block those changes and correlate them with behavior. Immunoprecipitated chromatin from the POA of embryonic and neonatal male and female rats was assessed for H3 and H4 acetylation in the dominant brain promoter for *Esr1*, promoter 1b, and the brain-specific aromatase promoter, 1f, as well as the gonadal promoter for aromatase, promoter II, which is also active in brain. A varied profile of relative acetylation between the two histones, the two ages, and the two sexes emerged, highlighting the dynamic nature of epigenetic modifications in the brain and hinting but not establishing that these changes are involved in hormonally-mediated differentiation. A more causal connection is found again with the use of a pharmacological inhibitor of HDAC—in this instance, TSA infused directly into the brain on the first 2 days of life. As adults, males showed markedly reduced sexual performance. Most HDAC inhibitors available to date are not specific to subtypes, but antisense oligonucleotide-mediated inhibition indicated both HDAC2 and HDAC4 are required for normal masculinization of behavior. Further support for this view is the observation that both enzymes bound to the *Ers1* promoter, but only HDAC2 was found to be associated with the aromatase II promoter.[393]

Combined, these studies implicate epigenetics as an important component of the organizational, or enduring, aspect of brain sexual differentiation. The information to date is likely only the tip of the iceberg, and this will be an important and exciting area for future research.

Methylation of Steroid Receptor Gene Promoters in Brain

As the primary transducers of steroid action, developmental sex differences in ER and AR would be anticipated in those brain regions most impacted by organizational steroid effects and epigenetic programming as a means to regulate any observed sex differences. There are some modest sex differences in the levels of these steroid receptors, but both the magnitude and consistency are not sufficient to explain masculinization vs feminization of the brain. This is self-evident in the demonstration that giving testosterone (or estradiol) to newborn female rodents will fully masculinize many aspects of brain and behavior, thereby confirming they have the requisite receptors. Nonetheless, there are age, species, and sex differences in the amount of steroid receptors in various brain regions, which presumably underlie some functional outcome. Perhaps the most dynamic range of ER expression is found in the cortex where levels are quite high in the very young brain but decline with advancing age. The change in expression levels is nicely paralleled by changes to the methylation status of the promoter of *Esr1*, which increases with age as receptor expression declines. Both males and females show similar patterns of high to low *Ers1* expression over the course of development.[394–396] A re-emergence of *Ers1* expression following injury in the adult brain also appears to be the result of a removal of the repressive CpG methylations.[397] In contrast to the developmental regulation of ERα, ERβ is epigenetically regulated in the aging brain.[398] The intrinsic signals that direct these divergent patterns are unknown and their identification will be of considerable interest.

Although there are sex differences and developmental changes in ER expression in the POA, they are much more subtle than those in cortical brain regions. Nonetheless, in rats there appears to be at least some degree of epigenetic regulation, as there is a developmental increase in the methylation of a portion of the *Ers1* promoter, with an almost doubling between birth and 3 weeks of age; however, there is no clear decline in receptor expression.[142] Thus, in this instance methylation marks may reflect past transcriptional history as opposed to future expression potential. What was perhaps most striking in this study, however, was the transient nature of observed sex differences in all three steroid receptors examined (ERα, ERβ, and PR), with some disappearing over time and other new ones emerging, suggesting a far higher degree of dynamism than previously anticipated (Figure 52.21). Examination of another portion of the promoter by a different group found good correlation between methylation levels and ERα expression in the POA of 10-day-old rat pups, such that males had higher methylation and lower ER levels and masculinization of females with estradiol treatment on the day of birth mimicked the profile of males.[278,399] A similar correlation is found between promoter methylation and AR expression in the cortex in that males have more AR and less methylation.[400]

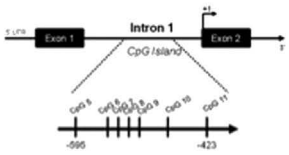
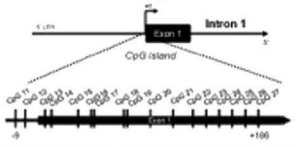
Gene of Interest	Brain Region	Postnatal Day 1	Postnatal Day 20	Postnatal Day 60
Estrogen Receptor α 	POA	Site 7: F > M, F+E2 Site 9: F > M, F+E2	No differences	Site 5: F > M, F+E2
	MBH	Site 6: F > M Site 9: F > M	No differences	No differences
	HIPP	--------	No differences	No differences
Estrogen Receptor β 	POA	Site 5: M > F (p=0.07)	Site 6: F+E2 > M	Site 5: M > F, F + E2
	MBH	No differences	Site 6: F < M, F+E2	Site 4: F > M, F+E2 Site 5: F > F+E2 > M
	HIPP	No differences	No differences	Site 4: F+E2 > M, F
Progesterone Receptor 	POA	Site 11: F+E2 > M, F Site 12: F+E2 > F	No differences	No differences
	MBH	Site 13: F+E2 > M, F	Site 11: F < M, F+E2 Site 13: F < M, F+E2 Site 17: F < M, F+E2 Site 27: F+E2 > M, F	Site 27: F+E2 > M, F

FIGURE 52.21 Epigenetic changes to steroid receptors in brain are highly dynamic. Pyrosequencing of bisulfite converted DNA was used to compare the methylation status of a small region of the promoters of the genes encoding for ERα, ERβ, and the progesterone receptor (PR) in male (M) and female (F) rats that were 1 day, 20 days, and 60 days old in three brain regions: preoptic area (POA), mediobasal hypothalamus (MBH), and hippocampus (HIPP). An additional group of females was treated with a masculinizing dose of estradiol (F+E2) to determine if any sex differences in methylation observed were the result of organizational hormone effects. There were significant sex differences and hormonal modulation of the degree of methylation at multiple CpG sites in the newborn animals, but these had largely disappeared by 20 days of age and in some cases were replaced by new sex differences in adulthood. The *PR* gene displayed a number of sex differences in CpG methylation status at 20 days, a period when gonadal steroid levels are low. These results are highly reliable due to the large number of animals per group and the statistical robustness of pyrosequencing, but their physiological significance remains unclear other than to illustrate the degree of plasticity in steroid receptor promoter methylation and the lack of a clear relationship between methylation status and gene expression. *Source: Reprinted from Ref. 141.*

In a merging of epigenetic influences of maternal care and endogenous sex differences, Auger and colleagues took advantage of the well-established fact that rat dams perform more LG on their male pups than the females. Based on work of Meaney and Champagne, reviewed above, this difference in maternal care would be predicted to have an epigenetically mediated functional impact. Simulation of maternal grooming of pups with a paintbrush allowed for standardization of care between males and females, increased the level of methylation in the promoter of the ERα gene, and correspondingly decreased expression levels of the receptor in females to that of males.[278] This nicely demonstrates a convergence between experiential variables, such as maternal care, and endogenous hormones, such as estradiol, to mediate the establishment and perhaps maintenance of sex differences in the brain.

Together, this handful of studies on the methylation status of steroid receptor promoters in the brain reflects two important things: (1) there is not always a clear relationship been methylation status at a particular site and expression levels; and (2) what is true for one developmental time point or brain region may not be true for another. As a third point, these studies reveal how little we know and how poorly we understand epigenetic regulation of steroid receptors in the brain, highlighting the need for further investigation into the multitude of variables that impact on both steroids and their receptors and, in turn, the role epigenetics plays in that impact as well as in responsiveness.

Sex Differences in MBDs in the Brain

An additional approach to elucidating how and where epigenetics impacts the establishment of sex differences in the brain focuses on regulatory factors that have the potential to impact on large numbers of genes. The MBD genes are a primary candidate and

the iconic *Mecp2* is indeed higher in the VMN and amygdala of newborn female rats.[401] These two brain regions are notable for their primary role in the control of female sexual behavior and a panel of social and emotional behaviors, respectively, many of which differ between males and females. One in particular is social play, which is expressed during the juvenile period and is found to occur at a higher rate and with greater intensity in males in a wide range of species, from laboratory rodents to domesticated pets to wild animals, humans and nonhuman primates. One of the more striking aspects of the sex difference in social play (also referred to as rough-and-tumble play) is that it peaks during a phase of life when steroid hormones are at their nadir. Thus, any sex differences that are hormonally determined were organized early in development but do not require activation—a scenario consistent with an epigenetic memory of earlier hormone exposure. Auger and colleagues have demonstrated via manipulation of MeCP2 levels in newborn rats that there is indeed an epigenetic programming of social play by hormones (Figure 52.22), and that the neural mechanism involves changes in vasopressin innervation.[401,402] Vasopressin has emerged as a central neuropeptide in a variety of social behaviors, including pair bonding, social recognition, and intraindividual aggression.[403–405]

Epigenetic regulation is emerging as the interface between the environment, experience, and the internal hormonal and metabolic milieu of the developing brain. Designed to program the brain to match both the reproductive capacity of the body and the anticipated external environment, these powerful changes can have lifelong enduring effects but also can be surprisingly transient and labile. This duality of purpose allows for both long-term anticipatory programming and short-term flexibility for altering course if the circumstances demand—precisely the qualities needed for successful reproduction. Further confirmation of this duality will come from study of additional species beyond the traditional rodent models, as reproductive strategies vary widely across the animal kingdom.

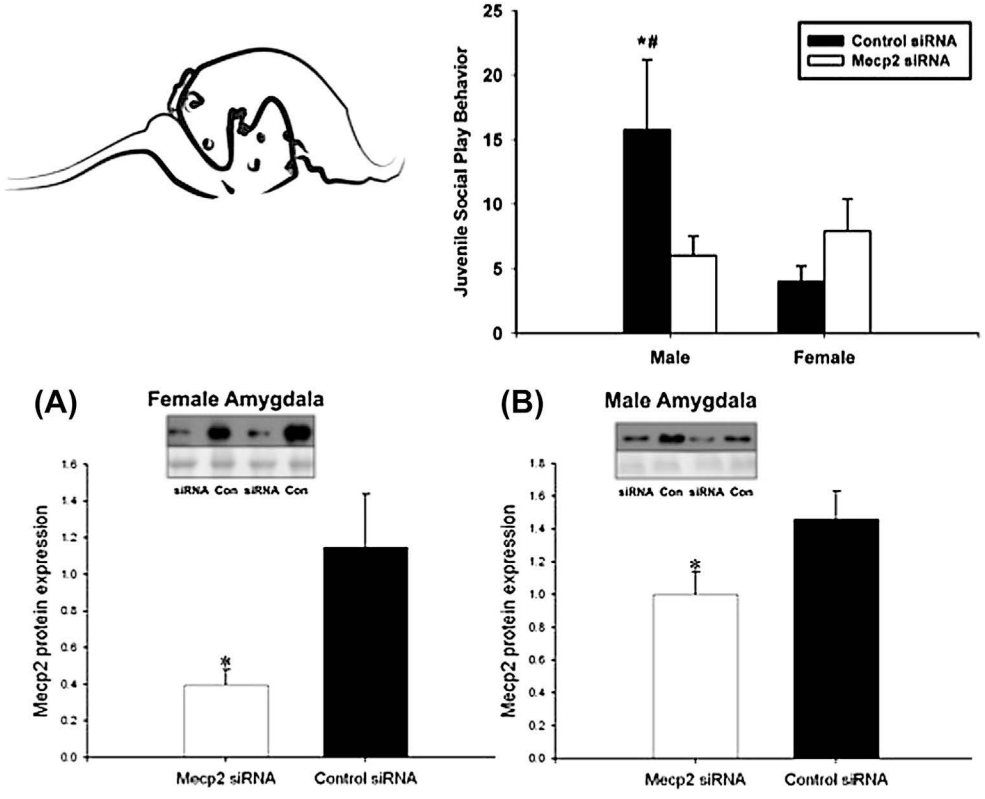


FIGURE 52.22 Epigenetics of social play. Juvenile animals of many species participate in play behavior with conspecifics. In rats, males play for longer duration and at a higher intensity than females, and this difference is determined by the organizing effects of perinatal gonadal steroids. Mecp2 is a methyl binding protein (MBD) originating from a gene on the X chromosome. Auger and colleagues administered a silencing RNA (siRNA) directed against Mecp2 mRNA into the amygdala of juvenile male and female rats. There was no effect on the already low level of play seen in females, but male play was significantly reduced compared to control injected animals (upper panel, *p<0.05 compared to females, #p<0.05 compared to Mecp2 siRNA treated males). The lower panel demonstrates the effectiveness of the siRNA at reducing Mecp2 protein in the amygdala and the lack of a sex difference in control animals (*p<0.05 compared to controls). These data indicate an epigenetic basis to the sex difference in social play but precisely how it is manifest remains unclear. *Source: Reprinted with permission from Ref. 401; drawing of playing rats courtesy of Anthony Auger.*

METHODS IN EPIGENETICS

The tools for studying epigenetics are many and rapidly growing. Because the principle enzymes in both DNA methylation and demethylation have now been identified, they can be pharmacologically inhibited, substrate limited or enhanced, genetically deleted, or overexpressed. Mice with single multiple or partial deletions have been generated for most if not all of the relevant enzymes, and work now is focusing on regional and temporal specific deletions as well as overexpression. This field is moving so rapidly that it is not feasible to review it in any meaningful way that would be of use to the reader. Instead, we will focus on standard methodology that is widely available.

Pharmacology

Most of the advances in drug targeting of either DNA or chromatin modifying enzymes come from cancer biology.[406,407] This has been tremendously beneficial as preclinical studies on toxicity, effectiveness, specificity etc. receive focused attention. Conversely, cancer cells as targets for drug manipulation are not necessarily the best model for the study of germ cells, early embryos, or the postmitotic cells of the brain and pituitary. Nonetheless, there is a reasonably sized tool set available for probing epigenetic questions in animal models, and this is only likely to increase as the promise of this approach in cancer biology and other biomedical disciplines grows.

Chromatin Modification

Inhibitors of HAT activity are not as numerous as inhibitors of HDACs, but this is an area of active investigation. The HAT inhibitors currently in use include several naturally occurring compounds. Polyisoprenylated benzophenone garcinol, a medicinal product isolated from rinds of the *Garcinia indica* fruit, inhibits both PCAF and p300.[408] Moreover, it can block the effect of at least one HDAC inhibitor. A more specific compound is anacardic acid from cashew nut shells. This inhibits p300, but in vivo it cannot penetrate cell membranes.[74] In LNCaP prostate cells, phenolic compounds from allspice can act as a HATi, producing decreased transcription of two AR target genes. AR co-activators are HATs and blockade of their actions may interfere with AR function.[409] Curcumin (diferuloylmethane), a natural polyphenol and popular spice derived from the plant *Curcuma longa*, is a very specific p300/CBP inhibitor and has biological activity in vivo.[410] In cells, curcumin can block the actions of an HDACi.[411] Curcumin also modulates activity of quite a number of miRNAs.[412] Although the mechanisms of action are under investigation, curcumin may target and disrupt Nf-kb signaling.[413]

The first synthetic HATi was a substrate analog for the acetyl CoA enzyme. Lysyl CoA is a p300 HAT-specific inhibitor. One issue with this and related molecules is cell permeability.[74] Compounds with an α-methylene-γ-butyrolactone core structure selectively inhibit GCN5. This drug has been used in cell lines and human tumors and may be useful for some leukemia treatments.[414] Isothiazolones are also able to block HAT activity in tumor cell lines by inhibiting PCAF.

Inhibitors of HDAC targets are being actively explored to treat diseases ranging from cancer to mental illness. In tumor cells, they act on cell death, migration, cell growth, and metastasis.[415] While chromatin modifications are ubiquitous, in fact, current HDACi only alter transcription in 2% of all genes.[416,417] Sodium butyrate is probably the most widely used HDACi. It is a short chain fatty acid that acts on all Class I and some Class II HDACs. In addition, it acetylates high-mobility group proteins and transcription factors and arrests cell proliferation.[418] Thus, off-target actions are an issue with this drug. Advantages of sodium butyrate include that it can be given in drinking water or by injection, making it very versatile.[419] This drug has also been used extensively for studies of epigenetics and brain function.[420] When provided to naïve female mice, sodium butyrate can enhance the onset of spontaneous maternal behavior.[421] In the presence of wild-type ERα, it can also accelerate the onset of receptivity in female mice.[422] A similar compound is VPA, which is currently used to treat epilepsy and bipolar disorders. If taken during pregnancy, it increases the risk of neurobehavioral disorders in children and rats.[423,424] In mice, it alters sexual differentiation of vasopressin in the BNSTp.[375] TSA and suberoylanilide hydroxamic acid inhibit Zn(II)-dependent Class I and II HDACS. Both drugs have been used in vivo and in vitro. For a more in-depth description of HDACi biochemistry, see Ref. 425.

DNMT Inhibitors

The development of inhibitors of DNMTs stemmed directly from the observation that many tumor-suppressing genes became hypermethylated and thereby repressed, allowing for uncontrolled cell growth and division (i.e., cancer). The first to be developed are the so-called substrate analogs because they mimic cytosine residues (Figure 52.23), and first among these were 5-azacytodine and its derivative 5-aza-2′-deoxycytodine.[427]

5-AZA

As cytosine analogs, the 5-Aza compounds are incorporated into the DNA during replication. However, the modified pyrimidine rings of the aza nucleotide block the methyl group transfer reaction and, in

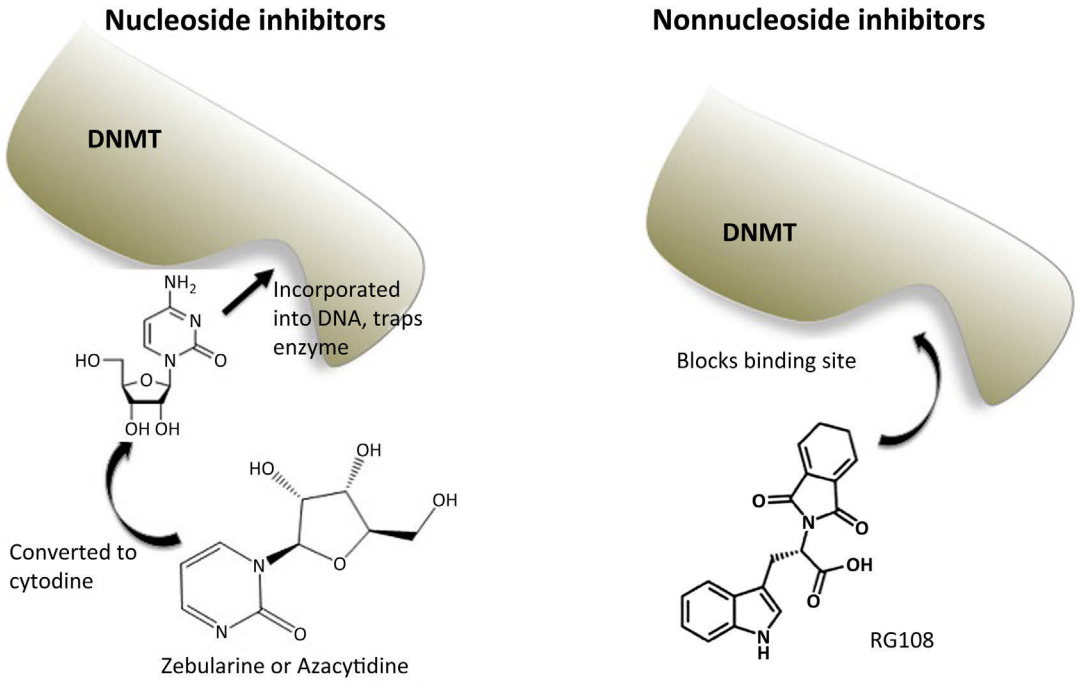


FIGURE 52.23 DNMT Inhibitors. Chemical inhibitors of the DNMTs come in two varieties: those that are nucleoside derivatives metabolized within the cell to a cytosine analog (cytodine) before being incorporated into the DNA (Zebularine and Azacytidine) vs nonnucleoside-based (RG108). Zebularine and azacytidine are ribonucleotide analogs and so-called suicide inhibitors in that they attract and covalently bind the DNMTs, which are then degraded, thereby depleting cellular stores of the enzyme and resulting in demethylation via as-yet unknown mechanisms. Nucleoside-based inhibitors are presumed to require cell division for incorporation into the DNA, but their effectiveness in nonmitotic cells, such as neurons, belies this requirement and suggests other mechanisms may be involved. Nonnucleoside-based inhibitors such as RG108 act in a more classic antagonist fashion and block the methyl binding site of the enzyme with direct involvement of the DNA. *Source: Based on references in Ref. 426.*

the process, trap the DNMT in a covalently bound DNA-protein adduct. This adduct is thought to be a major source of toxicity of the Aza compounds[428]; thus, questions have been raised regarding its specificity. Nonetheless, the 5-Aza compounds are still used experimentally and have been approved by the FDA for clinical trials.

ZEBULARINE

Zebularine (1-Beta-D-Ribofuranosyl-2(1H)-pyrimidinone) is, like Aza, a cytosine analog. However, it was developed to counter the shortcomings of Aza, in particular its cytotoxicity, instability, and short-half life. Zebularine is incorporated into DNA linearly over time; hence, it is attractive for use in rapidly dividing cancer cells.[429] However, this property was considered to make zebularine an unattractive agent in the nervous system, where most cells are postmitotic. Others argue that zebularine might stimulate demethylation via excision base repair mechanisms found in nondividing cells.[279] In cancer cells, continuous treatment for a period of days results in depletion of the DNMT enzymes, with DNMT1 being most sensitive. This is presumed to be due to the enzymes being trapped at the zebularine-containing DNA.[430] Thus, while the mechanism of zebularine action in cancer cells is well worked out, this creates a mystery for how it could also be effective in nonmitotic cells. In addition to its DNMT-inhibiting activities, zebularine blocks cytosine deaminase; in fact, this was the first action identified. This mechanism appears to involve at least in part a competitive inhibition of the enzyme. It is possible a similar action occurs in the brain only in the realm of DNMT activity, and that the unique regulatory control of DNA methylation in the brain may then result in pronounced demethylation, just as is seen in rapidly dividing cancer cells.[431]

RG108

There are still concerns with zebularin in regards to specificity and mechanism of action, which has led to the development of new compounds focused at the DNMT catalytic binding site. This has proven successful in the compound RG108, which is highly specific for DNMTs, has low toxicity, and has demonstrably reduced methylation in tumor cells.[432] The computer-aided drug design focused on DNMT1, but the high degree of homology and >50% amino acid conservation of the catalytic binding site of all of the DMNTs supports broad applicability of this drug.[433]

None of these drugs are thought to effectively cross the blood–brain barrier, which can be a considerable

barrier in empirical research of animal models, although some have had success with peripheral administration (see Ref. 373).

Measuring Histones and Their Modifications

Isolating Histones

Nuclear isolation is the first step in quantification of histones. This can be accomplished by cell lysis under hypotonic conditions. Next, nuclei are extracted in acidic or high-salt conditions. Acidic conditions are the standard, but new modifications might be isolated better with high-salt to protest against acid-induced degradation. In addition, acid-extraction can reduce yields by elimination of "free" histones not incorporated into the chromatin. Further purification can be accomplished with reverse-phase high-performance liquid chromatography.[434] Another method is to use alkaline extraction, which is faster than acidic extraction and may be better for some types of modifications.[435] Most of the methods have been developed with cell lines or yeast cells. In the case of the brain, we have found that homogenization of the brain by gently drawing and ejecting the material through a series of large to small gauge needles is preferable to using a tissue shredder.[392] Other protocols are also available, which use dounce homogenizers.[436]

Visualizing Histones

Histones are positively charged, low-molecular-weight proteins. Their PTMs also affect their charge. The five major histones—H1, H2a, H2b, H3, and H4—can be resolved with standard sodium dodecyl sulfate (SDS) polyacrylamide gels. However, this method is not sensitive enough to reveal modified histones. The best separation method for differentiation of known or novel modifications are triton X-100/acetic acid/urea (TAU)-polyacrylamide gels.[437] This method is more efficient than others providing better separation of histone proteins. However, the detergent in the gel impairs transfer to membranes; to circumvent this problem, an alkaline transfer buffer is used along with SDS, which displaces the TritonX-100.

Quantification of Specific Modifications

After histones are collected and transferred from SDS-polyacrylamide gels to membranes, modifications can be visualized with specific antibodies. Controls typically include total H3 or H4, as the total amounts of histone should be stable. Another good control is the cytosolic fraction, which should be histone-free. Densitometry programs are used to measure the relative amount of the modification.[436] Several companies have started offering kits that detect either total acetylation of methylation of H3 or H4 or specific modifications (BioCat). The authors have no experience with these products.

Associations between Chromatin and Specific Genes

ChIP is the most widely used method to examine protein–histone interactions. This method identifies genes associated with histone modification. The basic method is first to cross-link proteins with DNA using a fixative, typically formaldehyde. This freezes the interaction in time, which can be important in characterizing the dynamics of chromatin interactions. Next, sonication is used to mince the chromatin into pieces of uniform size, as required for the application. An alternative often used with brain tissues is micrococcal nuclease digestion. Then, chromatin is precipitated with selective antibodies bound to magnetic beads. The antibody used depends on the target. This same principle has been applied to DNA methylation using antibodies against 5-methylCytosine. It can also be used for other applications, such as transcription factor binding proteins associated with regulatory elements. Here, we will discuss its application for specific histone modifications. After the antibody-ChIP complexes are captured, they can be used to probe single genes or whole genomes (Figure 52.24). Overall, the major limitation for these techniques is the availability of highly specific antibodies. A comparison of the different ChIP approaches is presented in Figure 52.25.

Optimization of the technique for a specific tissue requires determining how long to fix the tissues, how much material to use, and how much antibody is needed. Positive controls would include samples with the modification of interest. A no-antibody control is required along with a nonreactive antibody (IgG). A portion of the original sample is also required to determine how much starting material was used. The simplest use of ChIP is to ask about interactions between modifications and specific genes. This is done by designing primers for RTqPCR, and then running ChIP or control material with or without (control) primers and examining amplification profiles. Given how little material is needed for RTqPCR, multiple target genes can be assessed in this manner. Another excellent control primer set would target regions of DNA not associated with transcription.

ChIP-on-Chip

This method was favored in the past, but now availability and price of whole-sequence analysis has largely replaced it. However, for animals that do not have a sequenced genome, or for researchers with a small number of candidate genes or interest in only one chromosome, ChIP-on-chip is still useful. In addition, a novel

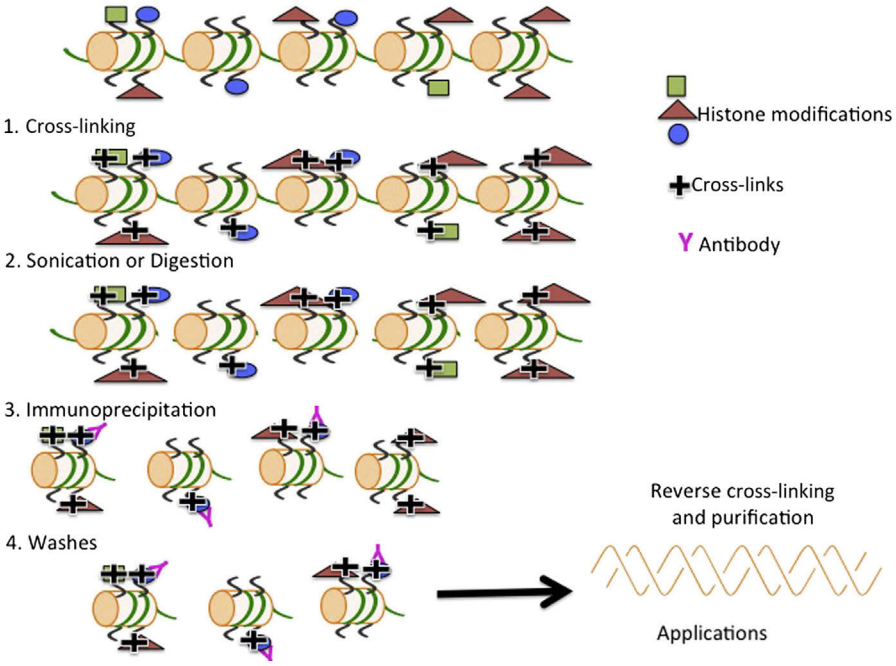


FIGURE 52.24 Chromatin immunoprecipitation (ChIP) methods. To perform ChIP, tissues are collected and rapidly exposed to fixative to cross-link the modifications and/or proteins of interest to the DNA. Next, sonification or enzyme digestion is used to produce uniform sized DNA fragments. Immunoprecipitation with an antibody for the histone modification is conducted, typically over antibody coated magnetic beads. A series of washes to remove fragments that are damaged is followed by confirmation that the material is the correct size. Various histone modifications are shown by circles, triangles, and squares. The cross-linking between the modifications and the DNA are shown with a plus sign. Antibody is designated by a "Y".

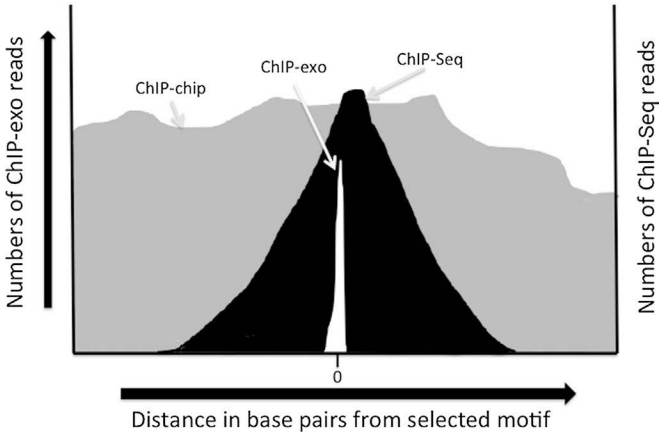


FIGURE 52.25 Comparison of ChIP methods. A theoretical comparison showing the increasing amount of specificity provided by less vs more refined methods for ChIP analyses. ChIP-chip = genome-wide microarray hybridization, Chip-Seq = deep sequencing, ChIP-exo = exonuclease is an extra step to trim the ChIP-DNA a precise number of exons from the linkage site. The technique can be used to target motifs of interest. *Source: Redrawn from Ref. 438.*

application of this technique is to combine it with a tiling array for miRNA promoters.[439] Moreover, this method is less complex than others in terms of data analysis. After ChIP DNA is purified, it can be labeled with different fluorescent colors and hybridized to a DNA microarray (also called tiling arrays). One problem for this technique is that DNA contamination may produce false positives. One solution to this is ChIP-exo. Here, an exonuclease is used to trim the ChIP DNA to a precise distance from the cross-linking site. Excess DNA is degraded.[438] Another downside to ChIP-on-chip is that typically only 10% of the DNA linked to protein is recovered and the amount of starting material (>500,000 cells/10–100 ng DNA) needed is more than it is for other ChIP applications. Amplification during the PCR reaction can always introduce biases. Finally, primer design can be problematic if the region on the gene of interest that interacts with the histone modification is not known.

ChIP Sequencing

Next-generation sequencing in combination with ChIP has become the criterion standard in the field. This method requires fewer steps than ChIP-on-chip, gives better genomic coverage, is more sensitive, and uses less starting material. The novel step as compared with other ChIP methods is the preparation of a genomic DNA library. Sequencing library preparation involves the production of a random collection of adapter-modified DNA fragments, with a specific range of fragment sizes, which are ready to be sequenced. This is accomplished by using blunt ended 5′ phosphorylated fragments to repair DNA fragments. Next, A-tails are added to the 3′ end of the fragmented DNA. The fragments are now able to be adapter ligated and are selected based on their size. PCR is conducted for amplification of the fragments, which are then made into single-stranded DNA. The DNA library is linearized, blocked, subjected to hybridization, and then sequenced. Florescent nucleotides are incorporated into the strand for the sequencing.[440] Many improvements have been made to this basic protocol, including development of nanoChIP sequencing.[441] This protocol only requires 10,000 cells and thus is well-suited for small brain regions and rare patient samples. One of the many improvements is use of custom-made primers that form a

hairpin structure at low temperatures to minimize primer self-annealing. Next, a second custom primer is used for PCR that produces DNA fragments with adapter-like sequences on both ends. The fragments can be ligated directly, thereby reducing the amount of amplifications and material associated with purification over columns. This method, combined with cell selection prior to DNA sonification, could be used to generate sequencing data from small numbers of neurons or other cell types. If florescence activated cell sorting is to be used to select cells of interest, then enzymatic digestion needs to be conducted after cells are dissected from the original material.[441] One common stumbling block, however, is that sequencing generates very large data sets. Bioinformatics programs are available (for a review, see Ref. 442), but for many investigators the optimal situation would be either a core facility specializing in bioinformatics or a collaborator.

DNA Methylation and Histones

Getting back to the idea that histone modifications can interact and that they are required for DNA methylation, we briefly review some techniques designed to interrogate these interactions. Using hierarchical clustering, Linghu et al.[443] identified the 18 most frequently occurring histone modifications in CD4+T human cancer cells. They also found that DNA methylation combined with histone acetylation more often than histone methylation. By labeling different modifications with different florescent dyes and subjecting them to single-molecule fluorescence resonance, energy transfer distances between modifications can be assessed. Thus, nucleosome constructs were methylated with a methytransferase enzyme and the relationship between modifications examined.[444] DNA methylation produced histone compaction and a repressive histone state. An interesting strategy to look at DNA methylation and chromatin cross-talk is sequential ChIP-bisulfite sequencing.[445] This approach can be applied to other histone modification, but the authors developed this to examine H3K27me3 and DNA methylation both of which are important components of the Polycomb repressive complex 1. Using this technique, the authors were able to settle a controversy on if and how often these two processes co-occur. They found abundant overlap between H3K27me3 and DNA methylation genome-wide; however, this was restricted to regions that did not contain dense CpG sites. In brief, they used ChIP for the histone mark DNA. End repair A-tailing and adapter ligation was performed and the material was isolated on agrose gels. Bands were collected based on their size and subjected to bisulfite conversion. Next, PCR amplification was conducted and material was used for sequencing. Several controls were included, such as MethylCap-Seq and in-depth analysis of 300-bp windows in the genomic data set.

Measuring CpG Methylation

Methods for the quantification of DNA methylation vary from those that can be readily performed in any reasonably equipped laboratory to those requiring extensive sequencing and bioinformatics support that only fully equipped centralized core facilities are likely to be capable of reasonably performing. Each has its advantages and disadvantages, and these shift in accordance with the question at hand.

DNMT Enzyme Activity

In the not-too-distant past, measuring DNMT activity was labor intensive, required the used of radioactive compounds, and was only semiquantitative. Fortunately, there are now kits available for measuring DNMT activity, which utilize a 96-well colorimetric based approach. These use an ELISA-based method in which the well is coated with cytosine-rich DNA that serves as a substrate for the enzyme and methyl groups are provided as donors; these are referred to as AdoMet, for SAM, and also referred to as SAM. The ELISA portion involves the use of recombinant MBDs that are His-tagged and attach to the methylated DNA in an amount proportional to the enzyme activity. An HRP-conjugated antibody against polyhistidine then provides a sensitive colorimetric readout, which is quantifiable by spectrophotometry. A sample of purified DNMT is provided as a positive control. A variation on this theme is provision of purified recombinant DNMT, which allows for determination of whether the factor is regulating DNMT1 vs DNMT3a for instance.

Global Methylation Approaches

The same general principle for DNMT activity can also be applied to quantification of DNA methylation without attention to specific sites. In this instance, the 96-well plates are antibody-coated to have a high affinity for DNA. The methylated portion of the DNA is determined by using capture and detection antibody based techniques as above, and the final readout is in optical density units which are proportional to the amount of methylated DNA as compared to a reference sample.

These techniques can be relatively quick and easy for initial analyses, but they are also lacking in details. The most powerful approach is to begin with these sweeping analyses followed by in depth and directed analysis.

Sequence-Specific Methylation Approaches

BISULFITE CONVERSION FOLLOWED BY SEQUENCING

Bisulfite conversion of DNA is an essential first step in most techniques for the detection of methylated cytosines (Figure 52.26). The basic principle is that when incubated in sodium bisulfite for an extended period, cytosines in

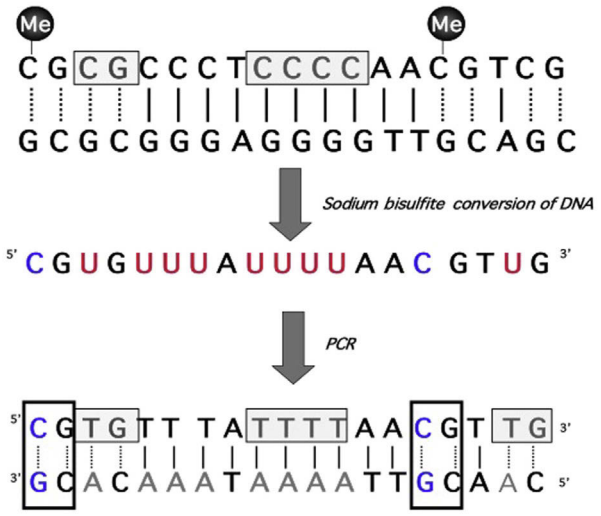


FIGURE 52.26 Bisulfite conversion. The central workhorse of most sequencing methods that detect and quantify 5mC is the conversion of unmethylated cytosines to uracil by bisulfite. Those cytosines that are methylated are protected from conversion. Subsequent PCR will convert the uracils to thymidines but faithfully replicate the previously methylated cytosines, although they lose the methylation during this process. Important limitations to the protocol are achieving a greater than 90% conversion rate and the inability to distinguish 5hmC from 5mC.

single-stranded DNA are deaminated to give uracil unless they possess a methyl group on the 5′ position, in which case they are protected from the deamination reaction. In order to detect which cytosines were protected after bisulfite conversion, the DNA is amplified by PCR and then either subcloned into a vector and multiple clones sequenced, or all the cDNA strands are directly sequenced via pyrosequencing, which is a sequencing by synthesis strategy. The "pyro-" in *pyrosequencing* refers to the release of pyrophosphate (PPi) following the incorporation of each nucleotide. The release of PPi occurs in an equimolar fashion to the number of nucleotides incorporated and is therefore highly quantifiable. The addition of ATP sulfurylase converts the PPi to ATP, which then activates a luciferase reaction and produces light in proportion to the amount of PPi generated. By controlling which of the four nucleotides is added at any given time, the amount of light generated provides a precise read-out of which nucleotide is incorporated, and how many are incorporated as the second DNA strand is synthesized. The disadvantage of pyrosequencing is that it is only effective for relatively short amplicons, 300–500 bp, but this is often sufficient for sequencing of CpG islands, which are rarely over 1000 bp in length. There are also restrictions on the primers that can be used, which may preclude comprehensive coverage of a particular island.

The major shortcoming of bisulfite sequencing is that it only works on single-stranded DNA and thus depends on complete denaturation and a lack of any annealing between the single stands. Thus, when a site is found not to be methylated, it is never completely assured that this is due to an artifact or lack of conversion by the bisulfite. An additional shortcoming of bisulfite sequencing is its inability to recognize hydroxymethylated cytosines, instead mistaking them for unmethylated, and they are deaminated to uracil. In many tissues, this may be a problem that is more apparent than real since presumably the majority of 5hmCs are fated to ultimately be converted to unmethylated cytosines. However, still-evolving views suggest these hydroxylated methyl Cs may be stable moieties conferring more information than previously realized ("the sixth base").

The quest for simple and accessible methods for DNA methylation analysis has spawned a cottage industry of techniques, which can be divided along four general lines: (1) use of restriction enzymes to detect sequence changes following bisulfite conversion, (2) PCR that is either impacted by methylation or builds upon it, (3) modified ChIP, and (4) brute force sequencing.

METHYLATION-SPECIFIC RESTRICTION ENZYME DIGEST

When the gene of interest is known, the promoter is well mapped with clearly identified CpG island(s), and the precise amount of methylation of a specific CpG residue is not necessary, the simplest approach may be the use of methylation specific restriction enzyme digest. This strategy is based on the simple principle that restriction enzymes recognize specific sequences of DNA and that when a CpG is converted to a uracil following bisulfite incubation, that recognition will be lost. Two PCR products, one from the original DNA and one bisulfite converted DNA, are subject to restriction enzyme digest, and the lack of a band produced from the bisulfite converted DNA indicates the CpG within the enzyme cite was methylated. A shortcoming of this approach is that it relies on a negative observation (i.e., no PCR product), and there is no quantification of the percentage of methylation. A modified version of this approach solves both of these problems with the generation of a standard curve that provides semiquantitative assessment of percent methylation and is dependent upon a positive result (i.e., cleavage of the PCR product to indicate either methylation or nonmethylation).[446]

METHYLATION-SPECIFIC PCR

Rather than depending upon sequence-specific restriction enzyme sites, methylation-specific PCR utilizes the specificity of primers which will only anneal to sequences that include methylated CpGs. This approach relies upon comparison to unmethylated PCR products (i.e., bisulfite converted), but avoids the need to sequence the PCR product since the sequence of interest is already

known. This approach is best when the density of methylated CpGs is high because it increases the specificity of the assay. As with restriction enzyme digest, there is no need to sequence the region of interest, but there also is no information provided on the absolute percentage of CpG methylation at a specific site or the degree of methylation of individual CpGs.

A variation on MSP is MethylLight PCR, which uses real-time PCR and therefore provides a quantitative analysis. In this instance, methylated-specific primers are again used, but a methylated-specific fluorescence reporter probe that anneals to the amplified region is included. A methylated reference DNA provides for quantitation. Further specificity can be achieved by also quantifying and subsequently subtracting DNA that was not successfully bisulfate converted using an additional probe, in a process called ConLight-MSP. In essence, this modification prevents detection of false positives. Finally, MSP-amplified DNA can be further assessed using melting curve analysis (Mc-MSP) of the two PCR products produced with methylated- and unmethylated-specific primers. This has been proposed for use in the sensitive detection of low-level methylation.

A further methodological approach based on PCR is referred to as MS-SnuPE, methylation-sensitive single-nucleotide primer extension, which takes advantage of the primer extension method initially designed for analyzing SNPs, or single nucleotide polymorphisms. In this instance, primers are designed to anneal to the sequence up to the base pair immediately before the CpG of interest. The primer is then allowed to extend one base pair into the C, or the T if the unmethylated C was converted to U during bisulfite incubation. The relative ratio of C to T is then determined quantitatively, which can be done using radiolabeled or fluorescently labeled nucleotides, pyrosequencing, and more recently MALDI-TOF and a modification of HPLC.

METHYLATION-DEPENDENT IMMUNOPRECIPITATION

Based on the same principle as ChIP, MeDip differs by using a monoclonal antibody specific to methylated CpGs that binds to the DNA following its sonication into fragments, preferably of 400–600 bp in length. This is followed by the classic immunoprecipitation technique in which magnetic beads conjugated to anti-mouse-IgG are used to bind the anti-5mC antibodies which are bound to the DNA and thereby precipitated. The technique works best when multiple CpG sites are methylated.

The next steps can vary and include MeDip-ChIP, in which a fraction of the input and immunoprecipitated DNA are labeled with fluorescent cytosines (either red or green) and then hybridized to standard DNA microarrays (hence chip) to probe for presence and relative abundance of methylation. As with any microarray, there are limits to the approach, but this can be a useful means by which to compare relative methylation between samples.

METHYL-SEQ

The advent of next-generation sequencing technology is providing for unprecedented levels of genomic information, including detailed unbiased and representative mapping of DNA methylation status that does not require intervening cloning into plasmids.[447] Methyl-seq is deep sequencing combined with bisulfite conversion so that specific methylation sites can be identified. Rather than sequencing the entire methylome, this approach can also be used for a series of candidate genes (economy of scale does not justify these approaches for only one or two genes) in which the CpG islands are identified and reduced to 300–600 bp sections that can then be sequenced. The approach is highly quantitative in that all the sequences in a sample are read, providing accurate and unbiased estimates of methylation.

METHYLCAP-SEQ

MethylCap-Seq is a clever combination of immunoprecipitation and deep sequencing, leveraging the advantages of each. The procedure allows for genome-wide profiling of DNA methylation but first captures highly methylated regions by immunoprecipitating with antibodies for the methyl binding domain of MeCP2. The captured methylated DNA is further enriched by eluting through a salt gradient which stratifies the genome based on different CpG density, thereby allowing for cost-effective deep sequencing of the enriched fraction.[448]

REDUCED REPRESENTATION BISULFITE SEQUENCING

If genome-wide methyl status information is desired but sequencing of the entire genome is not (due to the obvious limitations imposed by expense and large data sets), an additional approach is reduced representation bisulfite sequencing, which is analogous to a shotgun sequencing approach developed for single nucleotide polymorphism, SNP, analyses. The method uses restriction enzyme digestion to provide a "reduced" but random representation of the genome, which can then be compared between samples.[449]

This brief overview is not intended as comprehensive and is likely to be out-of-date as quickly as its written, but hopefully nonetheless provides some semblance of the range and variety of techniques available for studying epigenetic processes.

CONCLUSION

This is an exciting time in the field of epigenetics and reproduction. New discoveries are appearing so quickly we found it a challenge to keep this review up-to-date, and

it will likely be in need of additions by the time of publication. Central to this is the development of new technologies increasing the ease and accessibility of epigenetic assays so that individual laboratories are now capable of exploring in greater detail the realm of epigenetic modifications. The limitations inherent in bisulfite conversion to detect methylation of cytosines and the shortcomings of antibody detection of chromatin modifications are likely to all but disappear in the next few years with emergence of direct detection techniques. These will prompt a reanalysis of much of what has already been done with surely some surprises and refinements. New pharmacologies will further enrich the available toolbox and encourage more research in the field. Moreover costs for some of these techniques continue to come down and will soon be affordable for even modest laboratories. Nonetheless, major advances have been achieved, enough to both provide a level of confidence and detail appropriate for review and enough to inform us of how much we still do not know.

How are epigenetic marks retained in the zygote and/or germ cells to allow for transgenerational inheritance? That some genes are imprinted (meaning expression is preferentially limited to the allele inherited from one parent) has been known for some time, but how this imprint is maintained consistently across the generations is unknown. That it is truly maintained is evident in that the same parent of origin is faithfully propagated across generations, as opposed to it switching randomly between the maternally or paternally derived allele. There are two waves of global demethylation—one in the early zygote stage and one at the formation of the germ cells—but how imprints are maintained is entirely unknown.

How many genes are imprinted and how can the number or pattern of imprinted genes vary by tissue or sex? For many years the number of imprinted genes consisted of a short and well-known list of only a few hundred. Reports exploiting new technologies and the power of mouse genetics suggest there are far more imprinted genes than originally realized and that these can vary by tissue, brain region, or sex, meaning more or fewer imprinted genes in male vs female offspring, and that the degree of imprinting can change across the lifespan. Both how and why there is such plasticity in genetic imprinting is not known.

How does X inactivation switch from being exclusively of the paternally derived to random? There are two periods during the lifespan when X inactivation occurs—one very early in the blastocyst stage when the paternal X is silenced in every cell, followed by a period when they are both active and then random inactivation thereafter. If randomness is lost (i.e., inactivation is skewed in favor of the paternal or maternal X), the individual suffers serious deleterious consequences. How the switch from skewed to random occurs is unknown, and why there is a requirement for random inactivation is equally unknown.

Do epigenetic marks mediate and/or maintain sexual differentiation of the brain? Steroids are poised to mediate epigenetic changes via their activation of nuclear transcription factors which associate with enzymes capable of modifying both the chromatin and the DNA. It has long been a mystery as to how early hormonal exposure of the brain can exert effects that endure into adulthood. The notion of a cellular memory induced by steroids is a reasonable picture, but one for which only the broadest brush strokes have been made.

How do EDCs and other environmental agents affect transgenerational change? Several EDCs have been shown to have long-lasting effects on gonads, germ cells and the brain. In rodents, the only mechanism that has received experimental attention is DNA methylation. Certainly that is part of the process, but studies examining histone modifications, miRNA, and piRNA are lacking despite the fact that in nonmammalian species all of these are involved in transgenerational inheritance.

How are epigenetic marks removed and what determines that? For the emerging field of neuroepigenetics, this is a most fundamental question. That epigenetic changes can be a component of neuroplasticity is anathema to the definition of epigenetics as enduring. Demethylation as a biological process has proven difficult to definitively establish, although the emergence of the TET enzymes is beginning to change that. Major progress can be expected in this area in the next few years.

Are transposons and ncRNAs scientific oddities or major regulators of reproduction? Transposons have contributed to both genetic diversity and the physical structure of chromosomes, but they are still largely viewed as parasitic marauders capable of doing great harm if not contained. Their overrepresentation on the X chromosome and regulation by the SOX family of transcription factors, however, suggests they may be doing much more than realized, perhaps even contributing to the delicate dance that must be coordinated between males and females for reproduction to succeed. The ncRNAs are equally mysterious but again central, playing vital roles in X inactivation and potentially in the germ cell transmission of deleterious life events to the next generation. In the arena of "ones to watch", this class of molecular messengers is front and center.

How do environmental stimuli integrate with the epigenetic control of ovulation in females? That the PcG of repressive markers must be released to allow for kisspeptin expression in the brain nicely established epigenetics as an effector of ovulation, a process that is highly variable and responsive to environmental cues. Establishing how these cues are transduced epigenetically will provide much needed insights into what is contributing to

the still diminishing age of onset of female reproductive development.

Does epigenetics impact male puberty? While we have learned a great deal about the role of epigenetics in the timing of puberty onset in females, the same cannot be said for males. Are the mechanisms the same? Are environmental cues coded in the same way by males or do they carry a distinct salience that is manifest as unique epigenetic changes in males? The lack of a clear external marker of puberty in males that is evident in females (vaginal opening/menarche) makes this a greater challenge, but it is nonetheless approachable.

Does epigenetics play a role in reproductive senescence? Reproductive potential in the human female is typically limited to only three or four decades of her lifespan. While the loss of viable oocytes is central to this phenomenon, there is also a neural component to menopause (see Chapter 37). Is there a loss of important epigenetic repressive or stimulatory marks that precede menopause? If so, which are the brain genes being regulated—those encoding for steroid receptors, kisspeptin, or GnRH? These important questions have not yet been addressed but surely will be in the near future.

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Color Plates

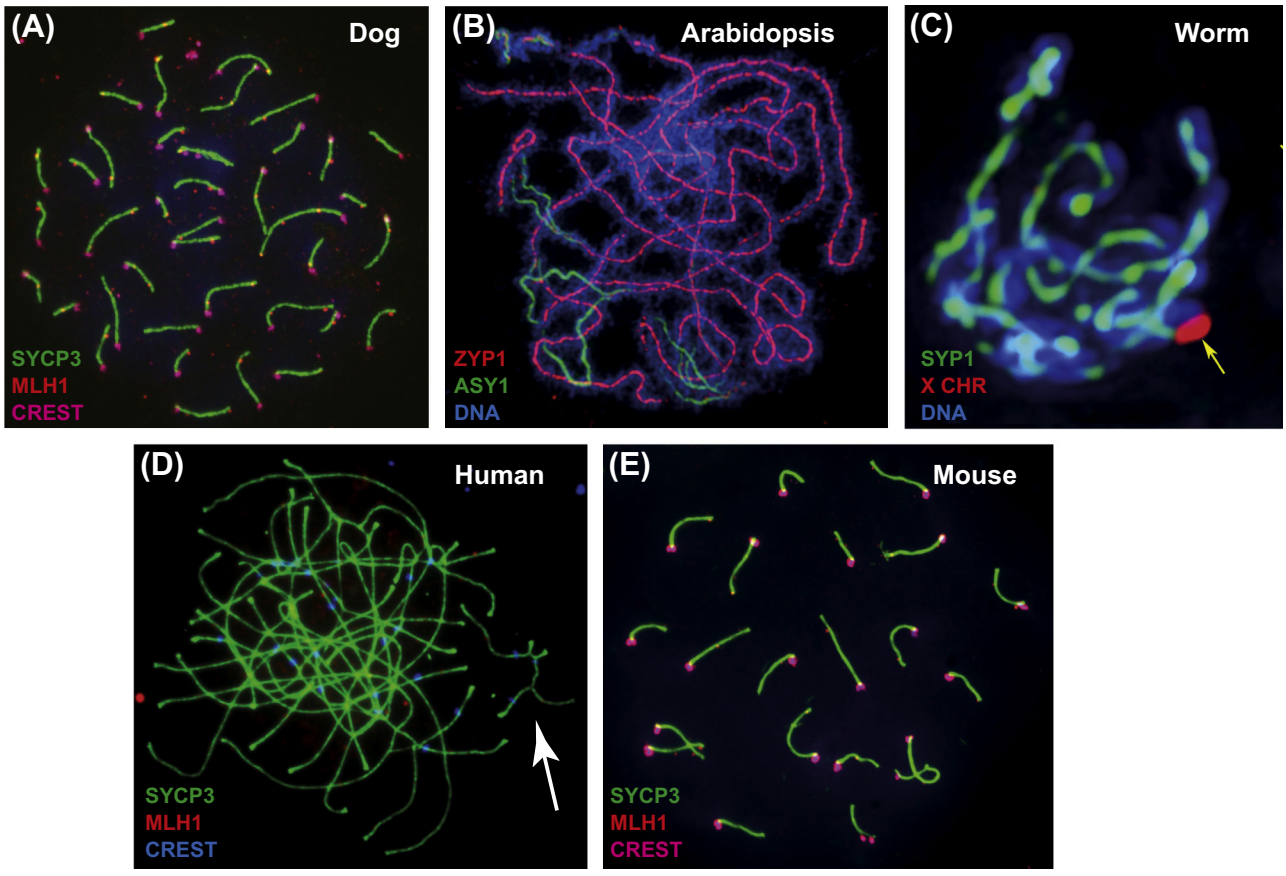

FIGURE 1.2 Comparative meiosis across eukaryotic species. (A) Pachytene cell from an adult dog testis stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, pink). (B) Immunolocalization of the *Arabidopsis* synaptonemal complex transverses filament protein ZYP1 (red), polymerizing between the homologous chromosome axes marked by ASY1 (green) and the DNA loops (blue) in a late-zygotene nucleus. The ASY1 signal (green) is reduced in synapsed regions (red), and an interlock is being resolved. (C) A *C. elegans* pachytene nucleus immunostained to show that the synaptonemal complex (green) is normal and that the pairing center ends of the X chromosome are fully paired (red); *(Source: Adapted from Saito et al., PLoS Genet, 2009.)* (D) A trisomic human embryonic oocyte at the pachytene stage stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, blue). The three copies of chromosome 21 can be seen attempting to pair (arrow). (E) Mouse pachytene cell stained immunostained with SYCP3 (green), MLH1 (red), and cREST (centromere, pink).

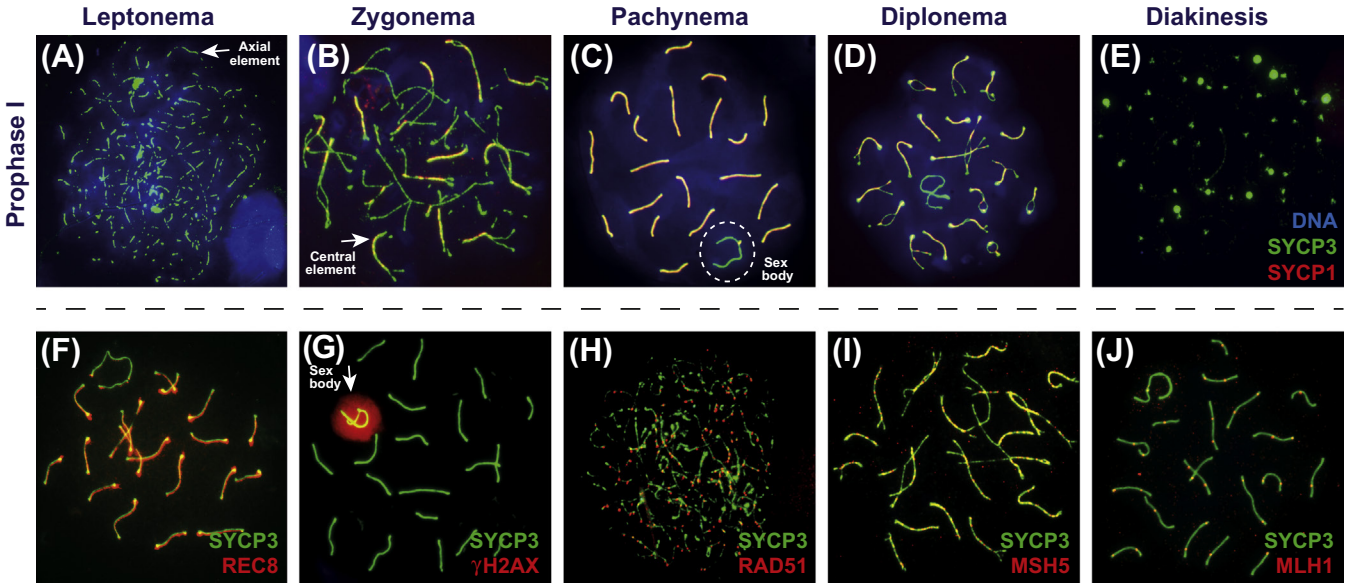

FIGURE 1.7 Immunofluorescent analysis of meiotic prophase I events in mouse spermatocytes. (A–E) Synaptic events in the five substages of prophase I, visualized by staining with antibodies against axial–lateral element protein SYCP3 (green) and central element protein SYCP1 (red), where the overlay between the two appears in yellow. (F–J) Immunolocalization of SYCP3 (green) with key meiotic markers during prophase I; (F) REC8, (G) λH2AX, (H) RAD51, (I) MSH5, and (J) MLH1 all in red, and SYCP3 in green.

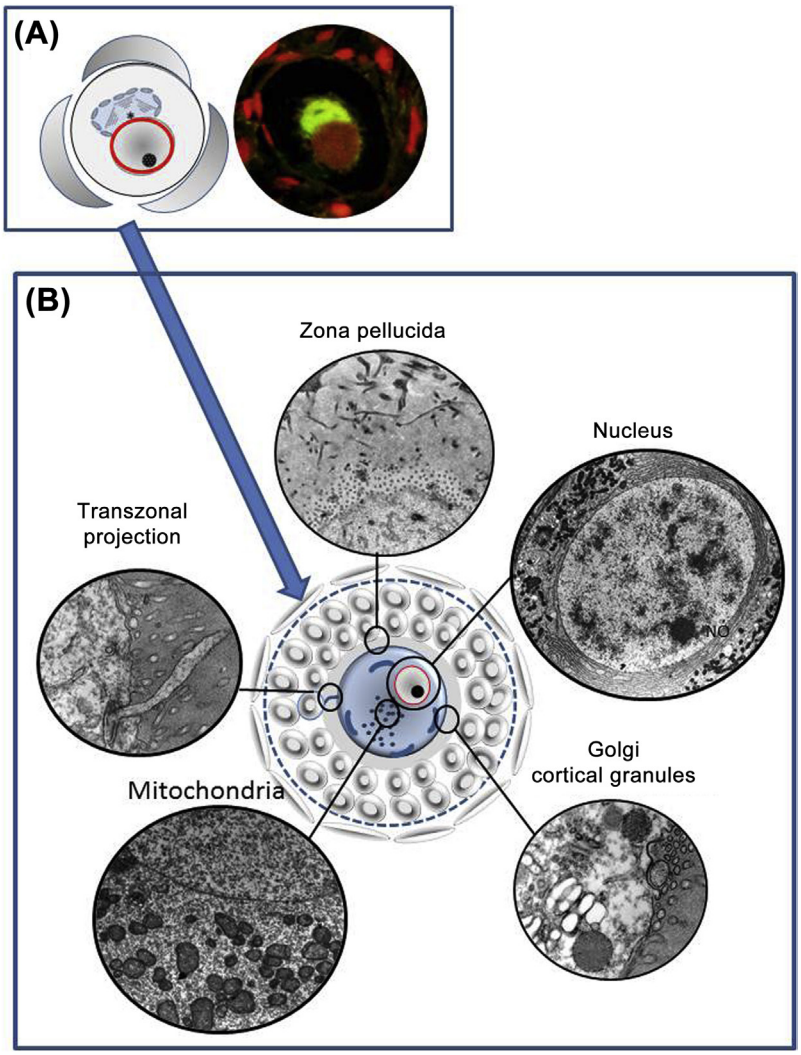

FIGURE 2.1 **Structure of the mammalian oocyte.** (A) depicts the general organization of organelles in a primordial follicle; schematic (left) denotes centrally positioned nucleus and aggregation of organelles known as Balbiani's body (asterisk). Right is an immunofluorescence micrograph illustrating the concentration of the germ cell–specific marker VASA within Balbiani's body (green) located next to the oocyte nucleus (red). *(Image courtesy of Professor Alfredo Vitullo)*. Upon activation, oocytes enter the growth phase of oogenesis (B). (B) summarizes the major ultrastructural features of a growing oocyte within a preantral follicle noting the assembly of the extracellular coat or zona pellucida, the enlarged nucleus or germinal vesicle with prominent nucleoli (NO), subcortical Golgi complexes with associated cortical granules, abundant perinuclear mitochondria, and the elaboration of microvilli on the oocyte plasma membrane interacting with somatic cell projections known as transzonal processes.

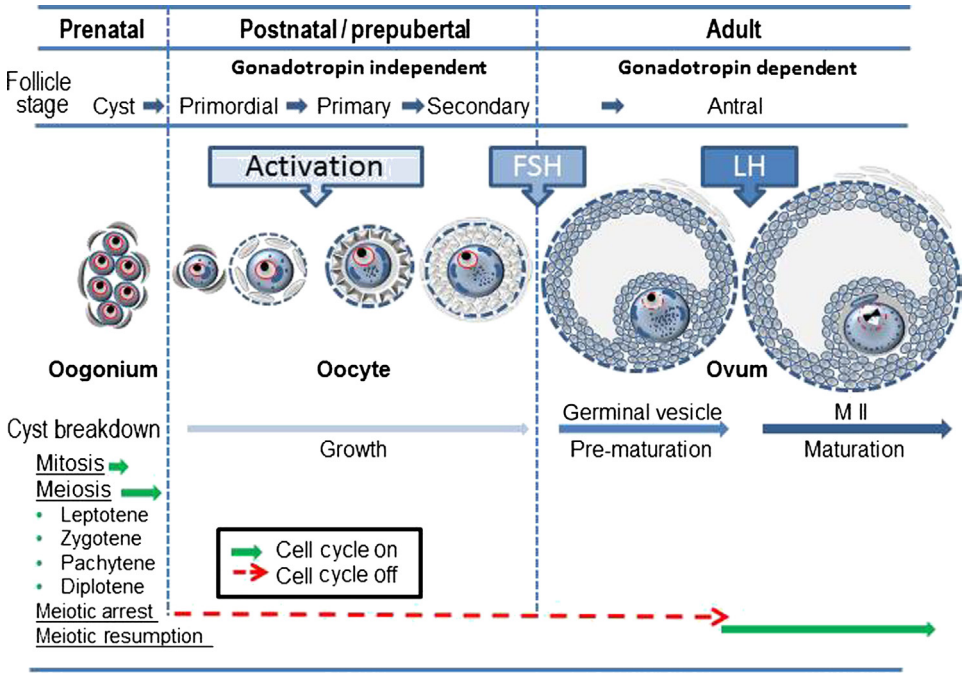

FIGURE 2.2 Stages of oogenesis defined on the basis of the oocyte cell cycle state as it occurs in the mouse. Mitotic proliferation of oogonia occurs in the prenatal gonad and is accompanied by entry into meiotic prophase as follicle formation and cyst breakdown take place. The cell cycle is then turned off for the duration of the growth phase of oogenesis that occurs during the preantral stages of follicle development. While FSH is required to advance follicle development, the oocytes remain in meiotic arrest with a germinal vesicle in the prematuration stage. In response to the LH surge, meiotic arrest is released and the oocyte proceeds through maturation being ovulated at the metaphase two stage of meiosis, which is maintained until the fertilization signal elicits completion of meiosis two. Note that in general the growth phase of oogenesis occurs independent of gonadotropin stimulation, whereas the prematuration and maturation phases require gonadotropin stimulation.

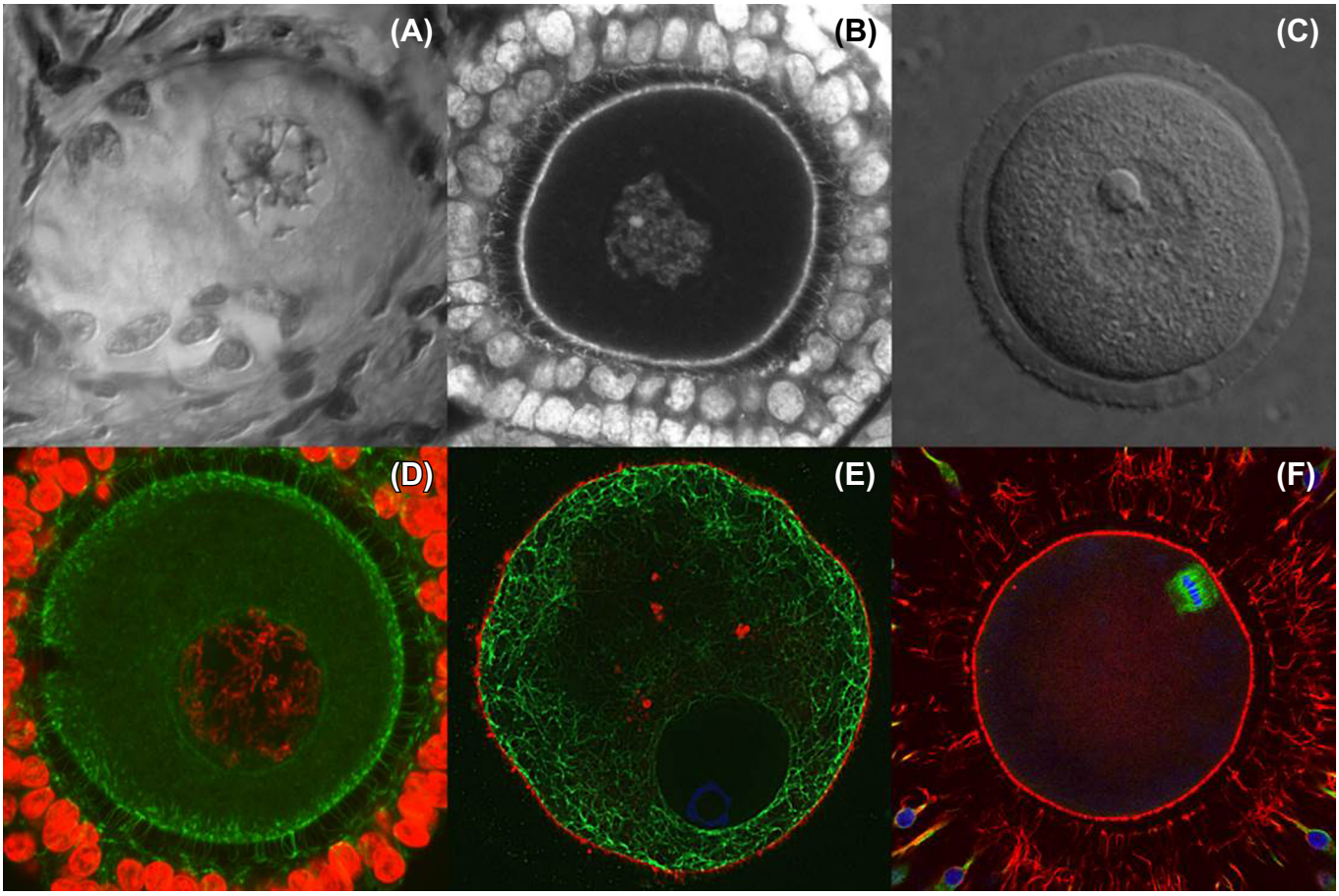

FIGURE 2.3 **Prominent morphological features of the mammalian ovum at various stages of oogenesis in various species.** (A) depicts a resting primordial oocyte within the bovine ovary in which the germinal vesicle chromatin assumes a distinctly fibrillar pattern of organization. (B) represents a primary growing oocyte from the mouse containing a centrally positioned germinal vesicle and illustrating the prominent actin-rich cortex and numerous transzonal projections emanating from surrounding granulosa cells. (C) demonstrates the appearance of a full-grown immature mouse oocyte following isolation from the ovary; note the prominent eccentrically positioned nucleolus within the GV and the cell free zona pellucida. (D) depicts a full-grown immature bovine oocyte isolated from a Graafian follicle; note the clear organization of bivalents in the GV and the presence of numerous foci at the oocyte cell surface, which represent terminal connections with corona TZPs (preparation labels f-actin using rhodamine phalloidin). (E) illustrates an immature GV-stage human oocyte that has been labeled for chromatin and microtubules (fibrillar structures); note that the chromatin is aggregated around the nucleolus of the GV and that a dense network of cytoplasmic microtubules is present in the outer regions of ooplasm. (F) illustrates a mature metaphase two arrested oocyte from a horse following controlled ovarian hyperstimulation; this preparation has been dual-labeled for tubulin (green) that highlights the cortical anchored meiotic spindle and f-actin (red) that labels the prominent corona cell projections or TZPs that appear detached from the oolemma.

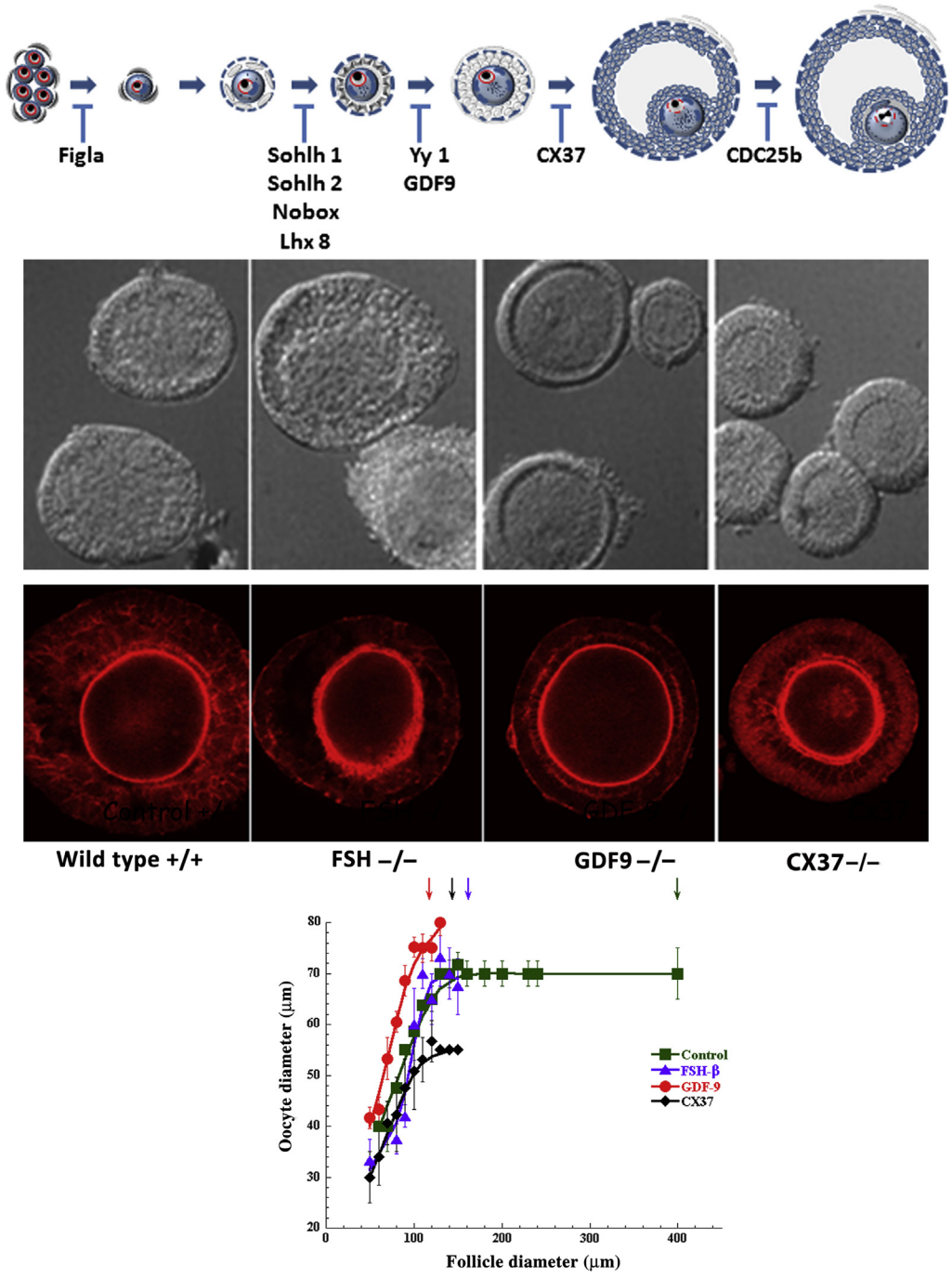

FIGURE 2.10 Mouse knockout phenotypes with distinct defects during the growth phase of oogenesis. Schematic at top shows effects of targeted gene deletion on stage of follicle development. Images show phenotype of wild type (left), GDF9, FSH beta, and CXN 37 in sequence that in the top panel show living follicles, and in the lower panel, the organization of granulosa cell–oocyte interface. Graph (bottom) plots the relationship between follicle and oocyte growth for the same four groups.

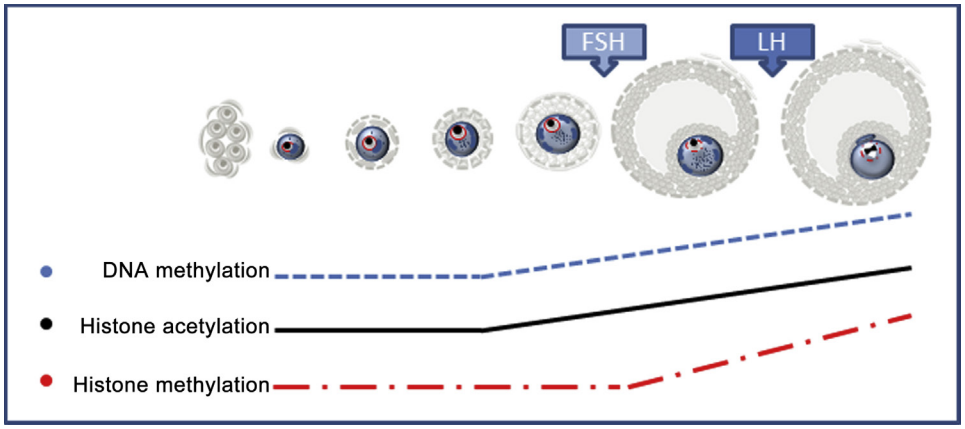

FIGURE 2.11 Epigenetic modifications of chromatin during the course of oogenesis. Genes subject to imprinting show progressive signs of DNA methylation beginning during the growth phase of oogenesis and ending at some point in the preovulatory follicle. Histone acetylation in developing oocytes follows a similar time course as DNA methylation, whereas histone methylation is reported to occur following the growth phase. Data are based primarily on studies in the mouse.

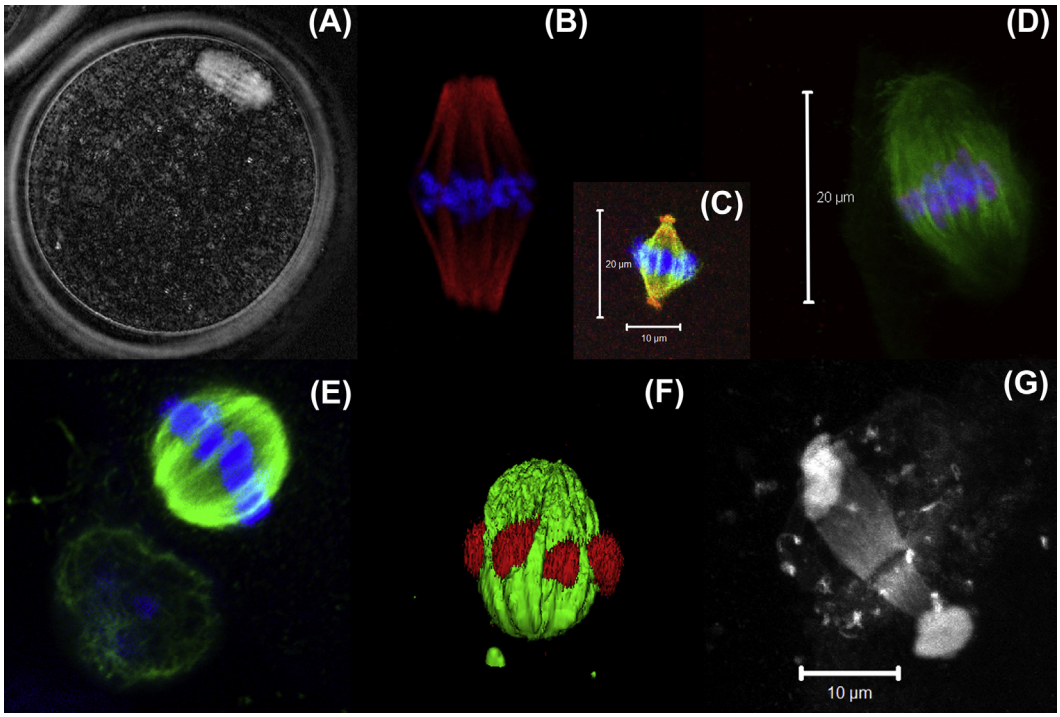

FIGURE 2.15 Meiotic spindles of mammalian oocytes. (A), (B), and (C) depict metaphase two–stage mouse oocytes under polyscope in a living oocyte (A), and after confocal immunofluorescence of tubulin (B, red; C, green); chromosomes aligned on the metaphase plate are rendered blue, and in (C), the poles of the spindle from a naturally ovulated oocyte show gamma-tubulin in red. (D) illustrates kinetochore microtubules as green stripes in a metaphase one horse oocyte that has been stained for chromosomes (blue) and phosphohistone H3 (red). (E), (F), and (G) are human oocytes at metaphase two (E and F, with the latter rendered as a three-dimensional projection); and (G) illustrates a projection of telophase one at the time of polar body extrusion.

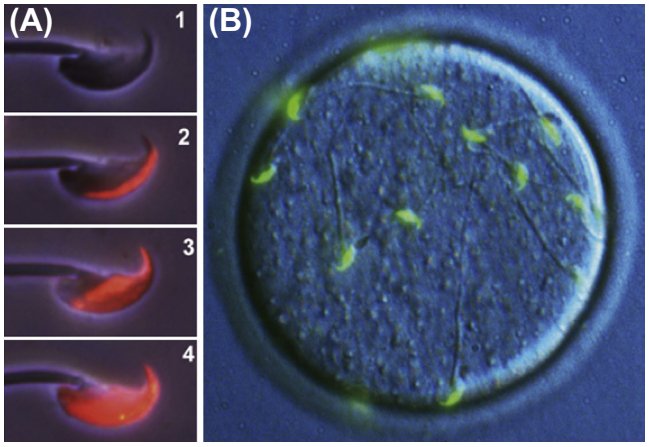

FIGURE 4.7 Gamete fusion-related factor IZUMO1 and localization in sperm. (A) IZUMO1 is an acrosomal membrane protein that is not exposed before the completion of an acrosome reaction (1). Acrosome-reacted sperm can be classified into three major groups by their IZUMO1-staining pattern: acrosomal cap (2), equatorial (3), and whole-head (4). (B) Accumulation of many sperm in the perivitelline space of the eggs recovered from females mated with *Izumo1*$^{-/-}$ males. Sperm in the perivitelline space were labeled with acrosome-reacted sperm-specific monoclonal antibody MN9. *Figure reprinted from Ref. 273 with permission from the* Asian Journal of Andrology. *Courtesy of Dr Masaru Okabe.*

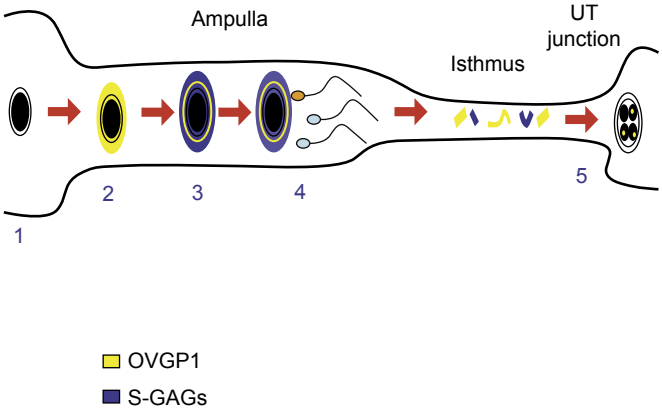

FIGURE 4.8 Depiction of the proposed role of oviductal contents in preventing polyspermy in ungulates. After the egg is shed into the ampulla following ovulation (1), oviduct-specific glycoprotein (OVGP1) surrounds it in a "shell" that renders the zona pellucida (ZP) resistant to proteolysis (2). Other oviduct molecules such as heparin-like glycosaminoglycans (S-GAGs) are thought to aid and strengthen the binding of OVGP1 to the ZP (3), modifying interaction with spermatozoa (4). As the zygote advances to the uterus, the aforementioned factors are shed or absorbed into the egg (5). *Figure reprinted and legend adapted from Ref. 375 with permission from John Wiley & Sons. Courtesy of Dr Pilar Coy.*

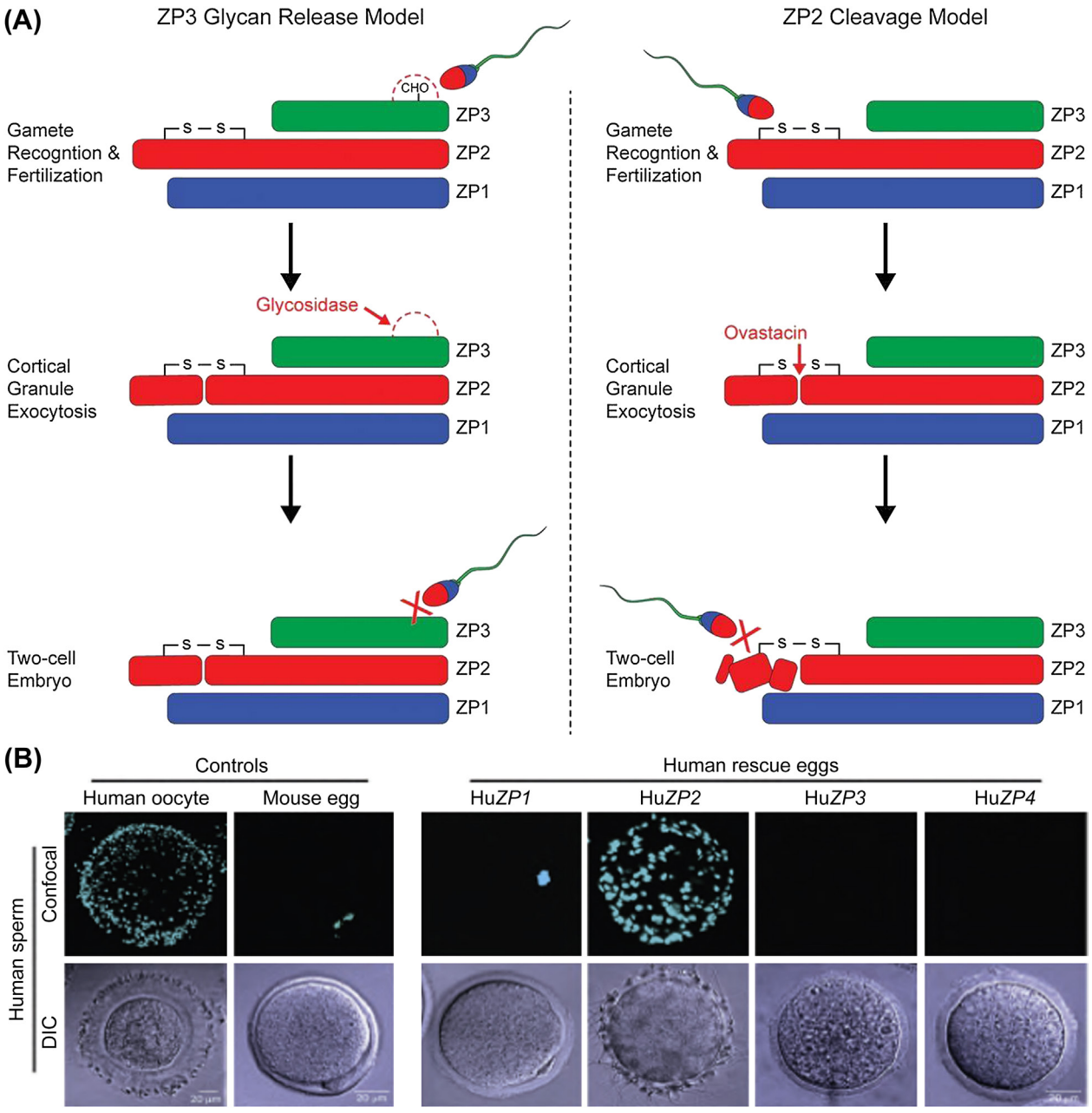

FIGURE 4.9 Models of zona pellucida block to polyspermy. (A) A specific glycan-release model (left panel) proposes that *O*-glycans on ZP3 Ser^{332} and Ser^{334} or possible others act as ligands for a sperm surface receptor. Following fertilization, cortical granules would release a glycosidase that would remove the *O*-glycans and account for the inability of sperm to bind to two-cell embryos. The ZP2 cleavage model (right panel) proposes that sperm bind to an N-terminal domain of ZP2. Following fertilization, ovastacin, a cortical granule metalloendoprotease, is exocytosed and the proteolytic destruction of the ZP2 docking site prevents sperm from binding to two-cell embryos. (B) Human sperm bind to mouse eggs from transgenic strains expressing human ZP2, but not other human ZP proteins, in the ZP. Confocal and differential interference contrast images of capacitated human sperm binding to the ZP of mice expressing human ZP1, human ZP2, human ZP3, or human ZP4, in the absence of the corresponding mouse protein. Human oocytes and mouse eggs served as positive and negative controls, respectively. Specific references and experimental details can be found in the text. *Figure reprinted from Ref. 229 with permission from Oxford Journals. Courtesy of Dr Jurien Dean.*

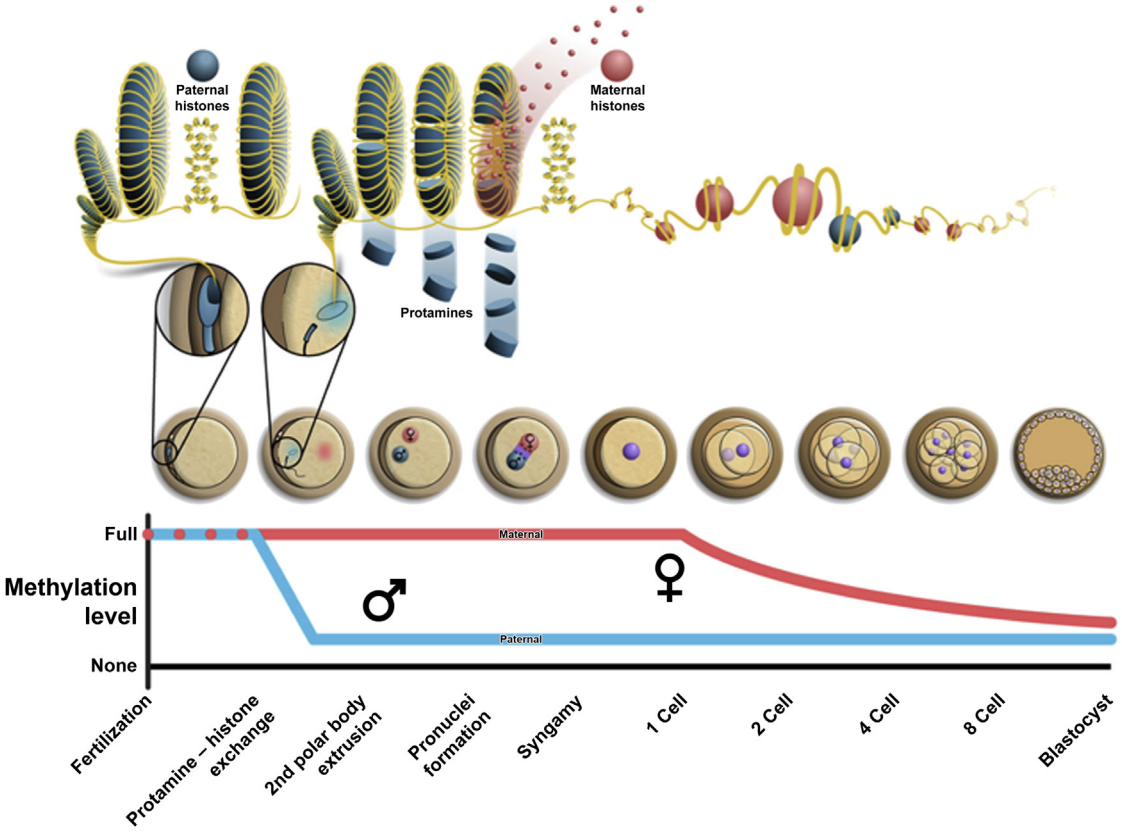

FIGURE 4.13 Reorganization of the male chromatin after fertilization and alterations to the epigenome postfertilization. The top panel illustrates the chromatin structure of the mature sperm immediately following fertilization (highly protaminated with some retention of paternally derived histones). Soon after sperm entry, the protamine to histone transition takes place where maternally derived histones replace protamines resulting in the decondensation of the sperm head. The middle panel illustrates the various stages of early embryonic development. The bottom panel shows the methylation changes that occur over time in the maternal and paternal pronucleus, where the paternal pronucleus undergoes active demethylation and the maternal DNA is demethylated passively in a replication-dependent manner. The approximate chronology of major events in the early embryo is outlined along the bottom of the figure and correlates to the illustrations of embryos above. *Figure reprinted from Ref. 444 with permission from Frontiers. Courtesy of Dr Douglas Carrell.*

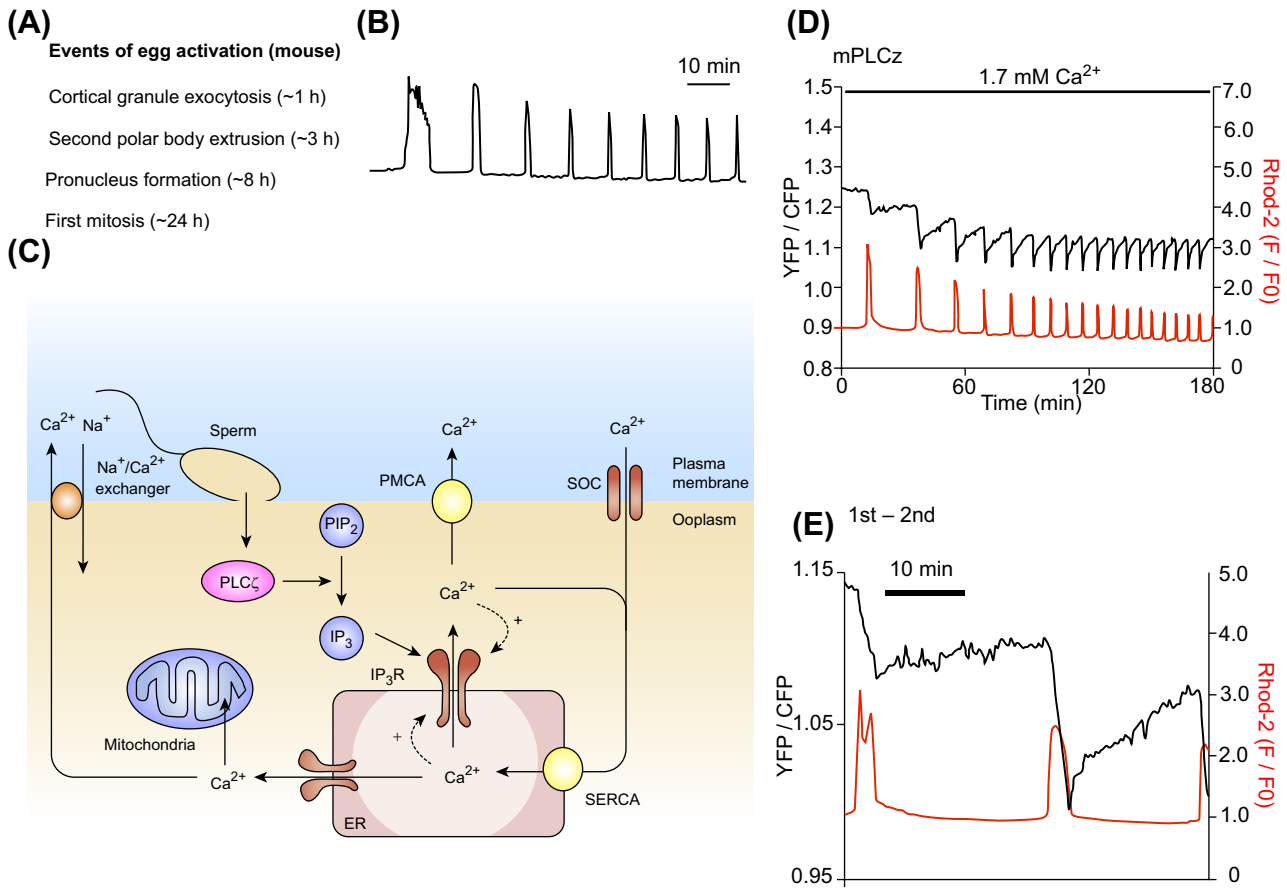

FIGURE 4.14 **Temporal course of activation events in mouse eggs with a characteristic Ca^{2+} response and candidate molecules involved in the initiation oscillations and in Ca^{2+} homeostasis.** (A) Main cellular events of egg activation after sperm entry and approximate time in hours (h) required for their completion. (B) A typical pattern of $Ca^{2+}{}_i$ oscillations associated with fertilization or with injection of PLCζ cRNA. Note that recordings were terminated prematurely. (C) Upon fusion, the sperm is thought to deliver phospholipase C (PLC) ζ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphospahte (IP_3) and diacylglycerol (DAG). IP_3 binds its receptor, IP_3R1, causing Ca^{2+} release out of the endoplasmic reticulum (ER). Following Ca^{2+} release, basal $Ca^{2+}{}_i$ levels are regulated by the combined action of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), plasma membrane Ca^{2+} pump (PMCA), Na/Ca^{2+} exchanger, and mitochondria. Store operated Ca^{2+} channels (SOC) or other Ca^{2+} channels are proposed to mediate Ca^{2+} influx, which is required to fill the ER and maintain oscillations. Broken lines suggest feedback action of Ca^{2+} on IP_3R1. *(Adapted from Ref. 507 with permission of CSHLP.)* (D) $Ca^{2+}{}_{ER}$ and $Ca^{2+}{}_i$ undergo simultaneous but opposite changes in concentration during oscillations in mouse MII eggs. $Ca^{2+}{}_i$ responses were induced by injection of 0.05 µg/µl mouse PLCζ cRNA into MII eggs. In vitro transcribed D1ER cRNA was injected into eggs 5 h before the initiation of $Ca^{2+}{}_i$ measurements. The emission ratio of D1ER (YFP/CFP) was used to estimate relative changes in $[Ca^{2+}]_{ER}$ (left axis, black trace). $[Ca^{2+}]_i$ (right axis, red trace) was recorded using Rhod-2. (E) The first and second $Ca^{2+}{}_i$ rises do elicit significant refilling of the ER, but subsequent rises, especially after ~ the sixth rise (D). *Adapted from Ref. 508 with permission from The Company of Biologists.*

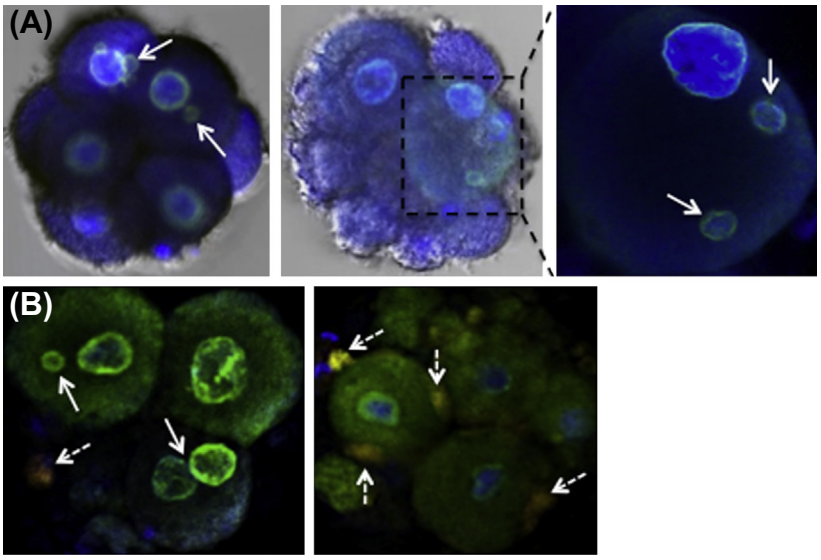

FIGURE 6.4 Detection of embryonic micronuclei in cleavage-stage human embryos. (A) Expression of the nuclear envelope marker, LAMIN-B1 (green), in 4′,6-diamidino-2-phenylindole (DAPI)-stained (blue) cleavage-stage human embryos visualized by immunofluorescent confocal microscopy and differential interference contrast (DIC) imaging at 20× (left and middle) and 40× (right) reveals the presence of several embryonic micronuclei (indicated by white solid arrows) distinct from primary nuclei in human blastomeres. (B) Similarly evaluated additional cleavage-stage human embryos with low (left) and high (right) fragmentation also immunostained for centromere protein E (CENP-E; orange) in the absence of DIC imaging identifies multiple missing chromosomes in cellular fragments (indicated by white dashed arrows) adjacent to blastomeres at this stage of development. *Source: Adapted from Ref. 16.*

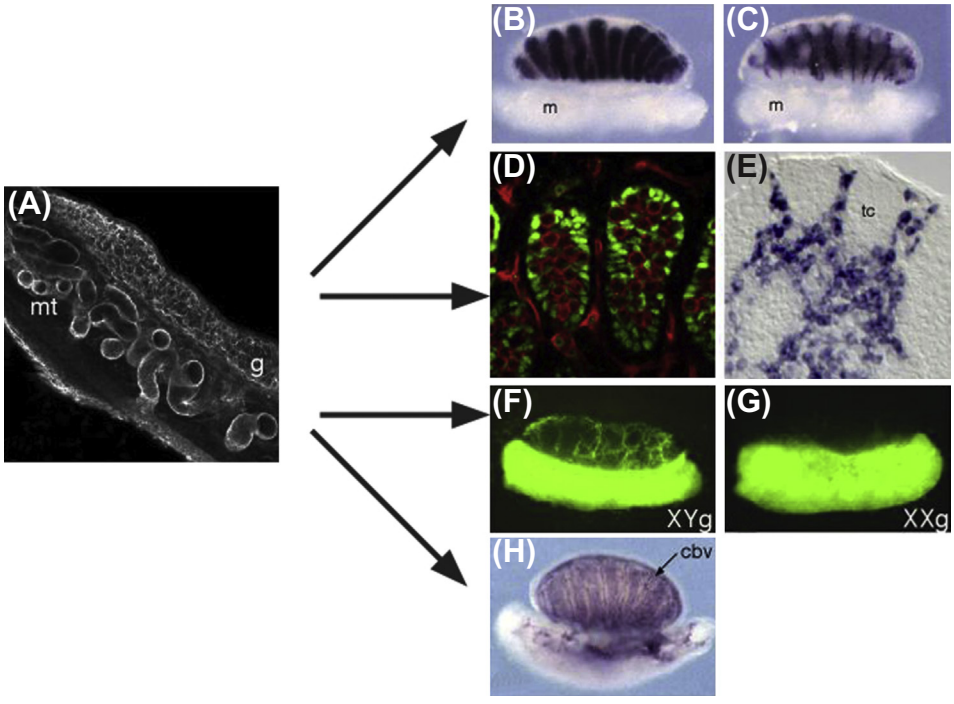

FIGURE 7.6 Differentiation of the embryonic testis from a bipotential precursor. (A) Confocal image of a gonad and mesonephros stained with an antibody to laminin at a stage before morphological changes take place (E11.5). (B–H) Morphology of the embryonic testis. (B, C) The Sertoli and Leydig cells of the E13.5 gonad are marked by whole-mount in situ hybridization for the expression of the Sertoli cell marker AMH (B) and the Leydig cell marker *Cyp11a1* (C). The structure of the testicular cords is seen in the confocal image of an E12.5 gonad that was stained with an antibody against SOX9 in green (D). Germ cells are found within the testicular cords as marked by the antibody stain for PECAM in red (D). In the interstitium, epithelial cells, as marked by the antibody stain for PECAM in red (D), and Leydig cells, as marked by the section of the whole mount in situ hybridization on a gonad from a E14 embryo for the expression of *Cyp11a1* (E), are found. (F, G) Mesonephric cell migration is observed in the XY (F) but not the XX (G) gonad, as shown by organ culture samples where the mesonephros but not the gonad is derived from transgenic mice that express GFP ubiquitously. (H) The formation of male-specific vasculature as shown by a whole-mount antibody stain to PECAM, which marks endothelial cells, of gonad and mesonephros from an E14 embryo. mt, mesonephric tubules; g, gonad; m, mesonephros; tc, testis cord; cbv, coelomic blood vessel. *Source: Adapted from Ref. 252; confocal image (D) courtesy of Blanche Capel.*

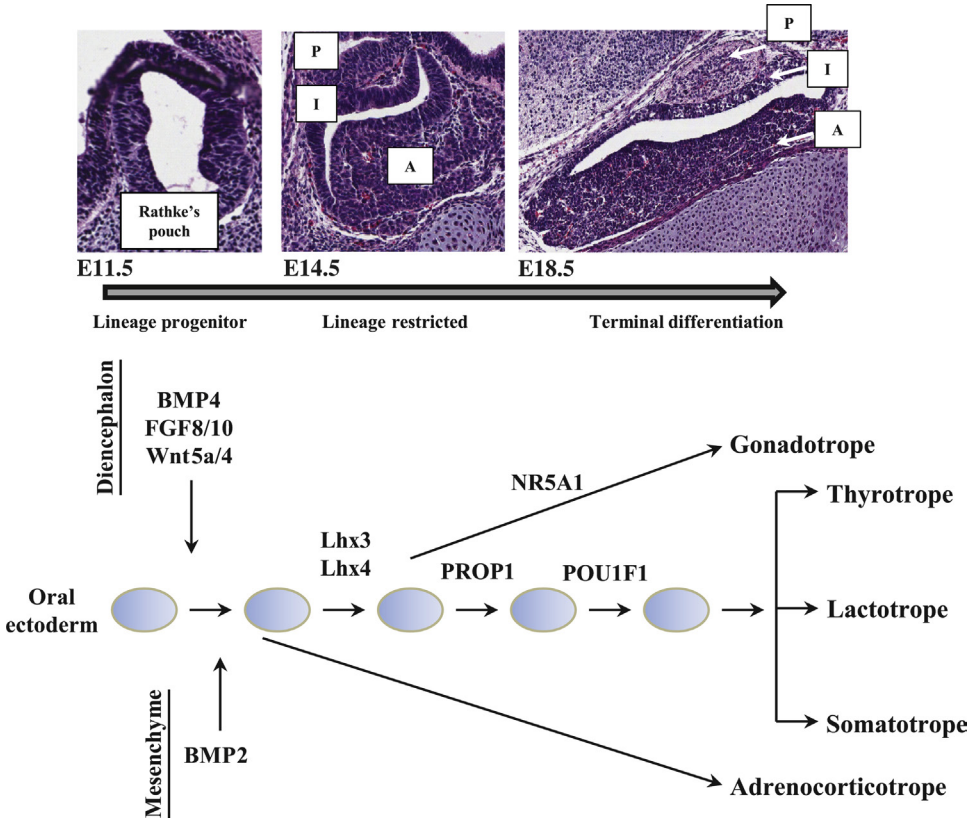

FIGURE 10.1 **Pituitary development.** The pituitary gland emerges from important interactions between the oral and neural ectoderm (diencephalon). Early expansion of the oral ectoderm appears to be functionally related to paracrine signals (such as bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs)) originating from the neural ectoderm and affecting proliferation and early differentiation of the oral ectoderm. The resulting expansion of tissue forms Rathke's pouch, the anlagen of the presumptive lineage progenitors of the anterior and intermediate lobes of the gland. In addition to signals emerging from the diencephalon, the surrounding mesenchyme provides additional paracrine signaling from BMPs, further restricting lineage identity. Differentiation of the five endocrine cell types within the anterior lobe of the pituitary is controlled by concise expression of a series of transcription factors that serve to restrict cell identity and drive terminal differentiation of the anterior pituitary. For specification of the gonadotrope cell lineage, early expression of Lhx3 (controlled by FGF signaling) and later expression of NR5A1 are central. Interestingly, Lhx3 and NR5A1 play central roles in the regulation of the four gonadotrope signature genes that define gonadotrope function in the adult animal. Sagittal tissue sections shown are from the mouse (unpublished data from M.S. Roberson and A.M. Navratil; e11.5 represents embryonic age in days post conception; P: posterior; I: intermediate; A: anterior).

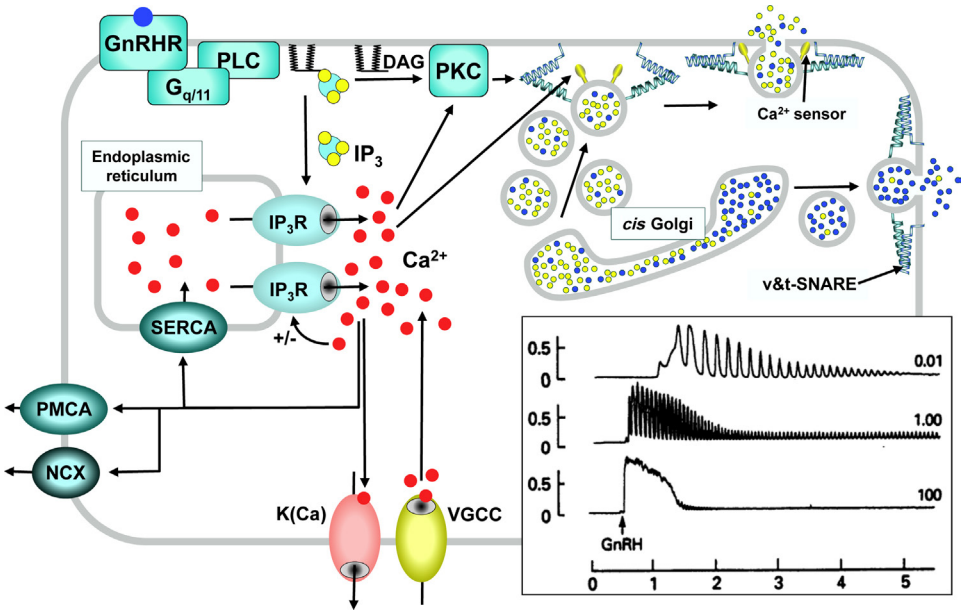

FIGURE 10.2 Acute effects of GnRH on Ca^{2+} and gonadotropin secretion. GnRH acts via Gq/11 coupled GPCRs to activate PLC, with consequent IP_3-mediated Ca^{2+} mobilization and Ca^{2+}–DAG-mediated activation of PKC. The interplay of Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry across the plasma membrane increases $[Ca^{2+}]_i$. This response may be oscillatory or more sustained, often with a spike–plateau-type profile. The response elicited by GnRH varies according to cell type and stimulus intensity but is also variable from cell to cell, even within isogenic cell populations. The GnRHR-mediated increase in $[Ca^{2+}]_i$ drives the secretion of LH and FSH by regulated exocytosis, a process likely involving interaction of a vSNARE (i.e., synaptobrevin) and a t-SNARE (i.e., syntaxin) as well as regulation by a Ca^{2+} sensor (i.e., synaptotagmin). PKC can also stimulate gonadotropin secretion, acting as a positive modulator of Ca^{2+}-driven exocytosis. In addition to regulated exocytosis, gonadotropins may be secreted via the constitutive pathway. The proportion of FSH (blue circles) secreted constitutively is higher than that of LH (yellow circles), and this could reflect sorting into distinct secretory vesicles formed at the Golgi. This principle is illustrated here by the presence of a Ca^{2+} sensor on LH-enriched vesicles but not on FSH-enriched vesicles. The inset shows μM $[Ca^{2+}]_i$ in individual cells stimulated with the indicated concentration of GnRH (plotted against time in min) and is adapted from Ref. 258.

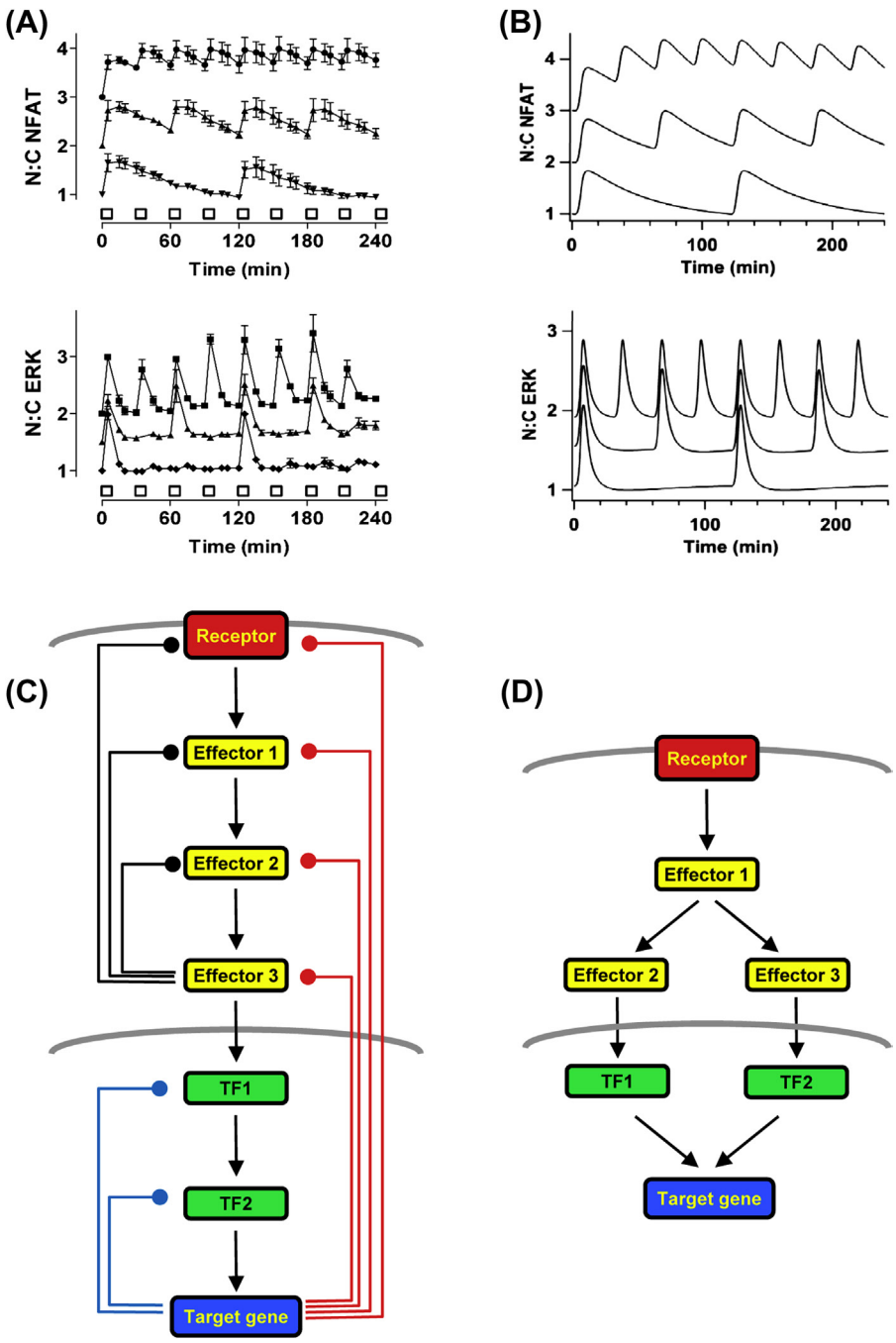

FIGURE 10.7 **GnRH pulse frequency decoding, part I.** Panel (A) shows the effects of pulsatile GnRH (1 nM pulses of 5 min duration and varied intervals) on the nuclear:cytoplasmic ratio (N:C) of NFAT2–EFP (upper chart) or ERK2–GFP (lower chart). In each case, the data are normalized to the prestimulation value and are offset on the vertical axis for clarity. Note that neither of the responses shows desensitization from pulse to pulse and that NFAT–EFP shows a cumulative sawtoothed response at the highest pulse frequency, whereas the ERK2–GFP response is not sawtoothed at any frequency (because it is rapidly reversed on removal of GnRH). Panel (B) shows data from an ordinary differential equation–based mathematical model that mirrors the empirical data for NFAT–EFP and ERK2–GFP translocation. Panel (C) shows a schematic signal transduction cascade in which a receptor in the plasma membrane causes sequential activation of three cytoplasmic proteins (effectors 1–3), and the last of these drives sequential activation of two transcription factors (TF1 and TF2) within the nucleus, which are upstream of a target gene. Also illustrated are multiple negative-feedback loops that could be restricted to the cytoplasm (black) or could involve transcription-dependent feedback to cytoplasmic proteins (red) or nuclear proteins (blue). Panel (D) shows a schematic network in which the receptor activates a bifurcating signal transduction pathway involving three cytoplasmic effectors and two nuclear transcription factors and where these transcription factors converge on the target gene. The empirical and modeled data in panels (A) and (B) suggest that the signal passing from the cytoplasm to the nucleus does not desensitize with pulsatile GnRH, arguing against the upstream negative-feedback pathways shown in black or red in panel (C). *Source: The data in panels (A) and (B) are adapted from Refs 618,620,621.*

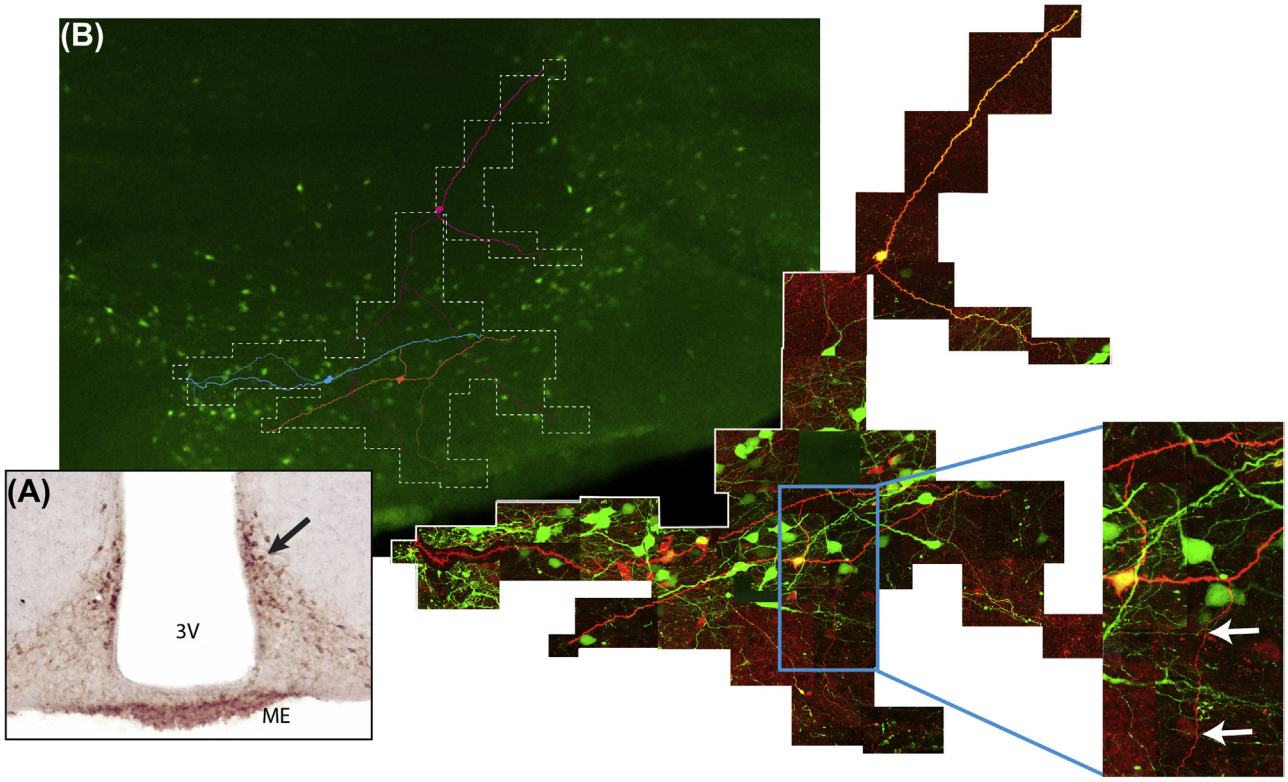

FIGURE 12.3 Neuroanatomy of the TIDA neurons. Panel A shows a coronal section through the mouse hypothalamus, with TIDA neurons identified by tyrosine hydroxylase (TH) immunoreactivity (3V: third ventricle; ME: median eminence). Note the cell bodies in the dorsomedial arcuate nucleus (arrow), as well as strong staining in the nerve terminals in the external zone of the median eminence. Panel B shows a low-power sagittal view of the hypothalamus from a mouse in which green fluorescent protein is expressed under the control of the TH promoter (green). These neurons were visualized in a brain slice, and then three individual TH-positive neurons were filled with neurobiotin (red; but pseudocolored pink, blue and orange in the low power view), allowing visualization of the full extent of the nerve processes. The montage (right) is constructed from multiple high-power views obtained using a laser confocal microscope, illustrating the dentritic tree of three filled cells extending a long distance throughout the nucleus. In the enlargement (right), note the major dendrites coming off the cell body, identified by a wide diameter and multiple spiny processes. The thin, smooth axon (arrow) arises off a primary dendrite and projects down toward the median eminence in the base of the hypothalamus. *Source: Images provided by Drs Michel Herde and Allan Herbison, based on work published in Ref. 86.*

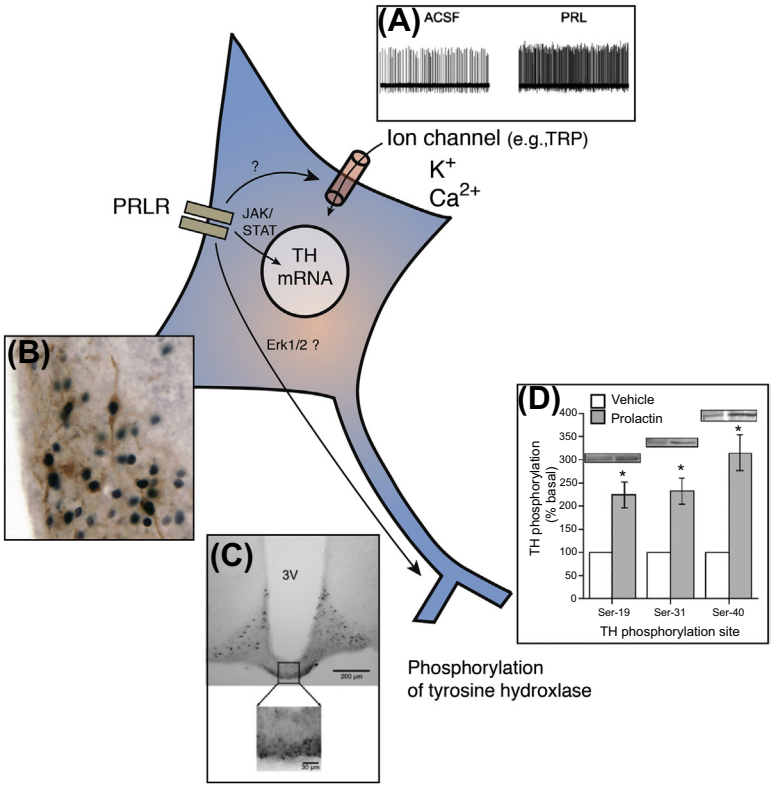

FIGURE 12.4 Diagrammatic representation of the multiple levels of prolactin feedback on NEDA neurons. Prolactin binds to prolactin receptors on NEDA neurons and then acutely (within 5 min) increases the firing rate of those neurons. Inset (A) shows prolactin-induced increase in the firing rate of a TIDA neuron.[92] Prolactin also increases the phosphorylation of STAT5 in TIDA neurons (approximately 15–20 min), inducing a transcriptional response. Inset (B) shows prolactin-induced STAT5 immunoreactivity (black) co-localized with immunoreactivity for tyrosine hydroxylase (TH) (brown), demonstrating direct prolactin action in these cells.[91] Finally, prolactin induces the phosphorylation of TH (approximately 15–30 min) both within the cell body and, more particularly, in the nerve terminals within the median eminence. Inset (C) shows prolactin-induced serine 40 phospho-TH immunoreactivity in the arcuate nucleus and median eminence (unpublished data); inset (D) shows prolactin-induced phosphorylation of TH at serines 19, 31, and 40 in mediobasal hypothalamic cultures.[102] ? indicates an uncertain mechanism; **P* <0.05.

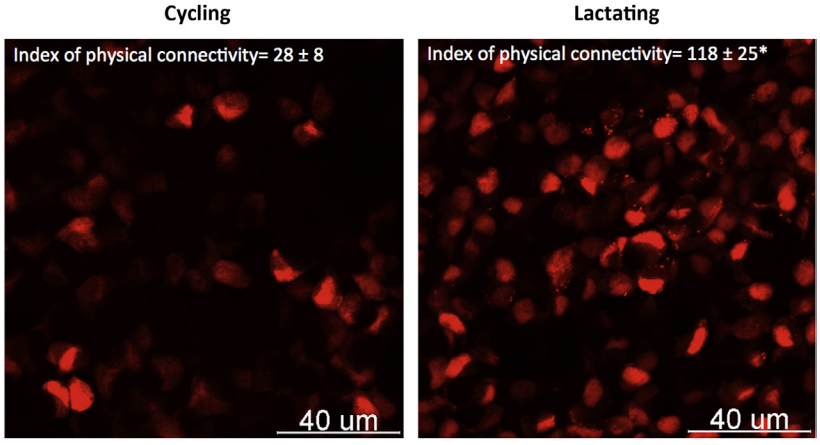

FIGURE 12.5 Biphoton imaging of pituitaries from virgin (left panel) and lactating (right panel) mice with transgenic expression of DsRedExpress fluorescent protein identifying lactotroph cells. In the virgin pituitary, lactotrophs are organized into a network of honeycomb-like structures. In the lactating pituitary, these honeycomb structures become more pronounced, and there is an overall increase in the proportion of lactotrophs with homotypic contacts. The "index of physical connectivity" is generated by three-dimensional reconstructions of the fluorescent label and reflects the degree of structural connections of lactotrophs with other lactotrophs. ? indicates an uncertain mechanism; **P* <0.05. *Source: Image courtesy of Chrystel Lafont, Taoufik El Yandouzi, and Patrice Mollard, Montpellier, France, based on work published in Refs 327,340.*

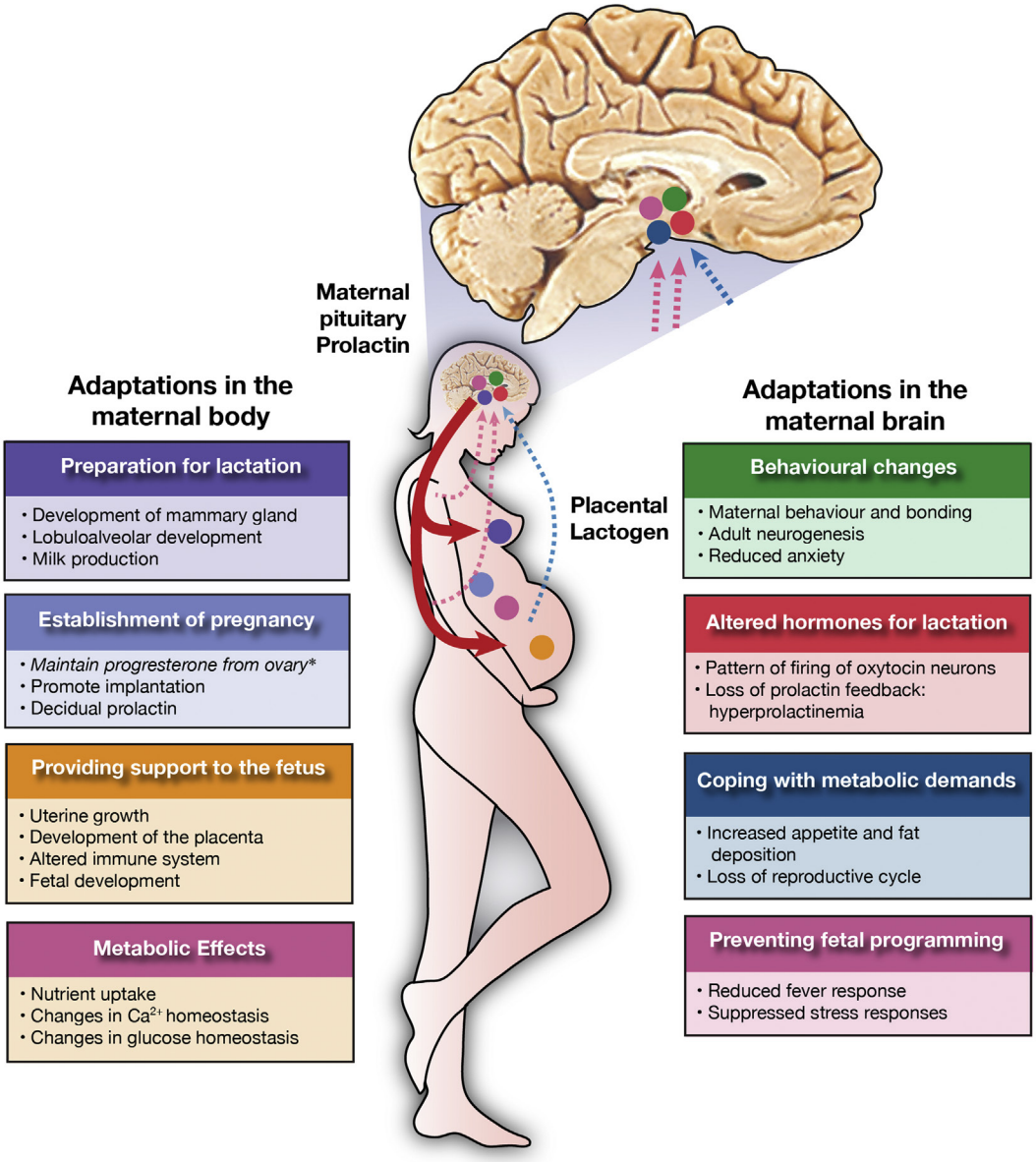

FIGURE 12.8 **Summary of adaptive responses in the pregnant female that may be induced or regulated by prolactin and/or placental lactogen.** Note that while this figure shows a human pregnancy, most of the data are based on experimental data from laboratory animals, and some have not yet been confirmed in women. *Those shown in italics are known to occur only in rodents.

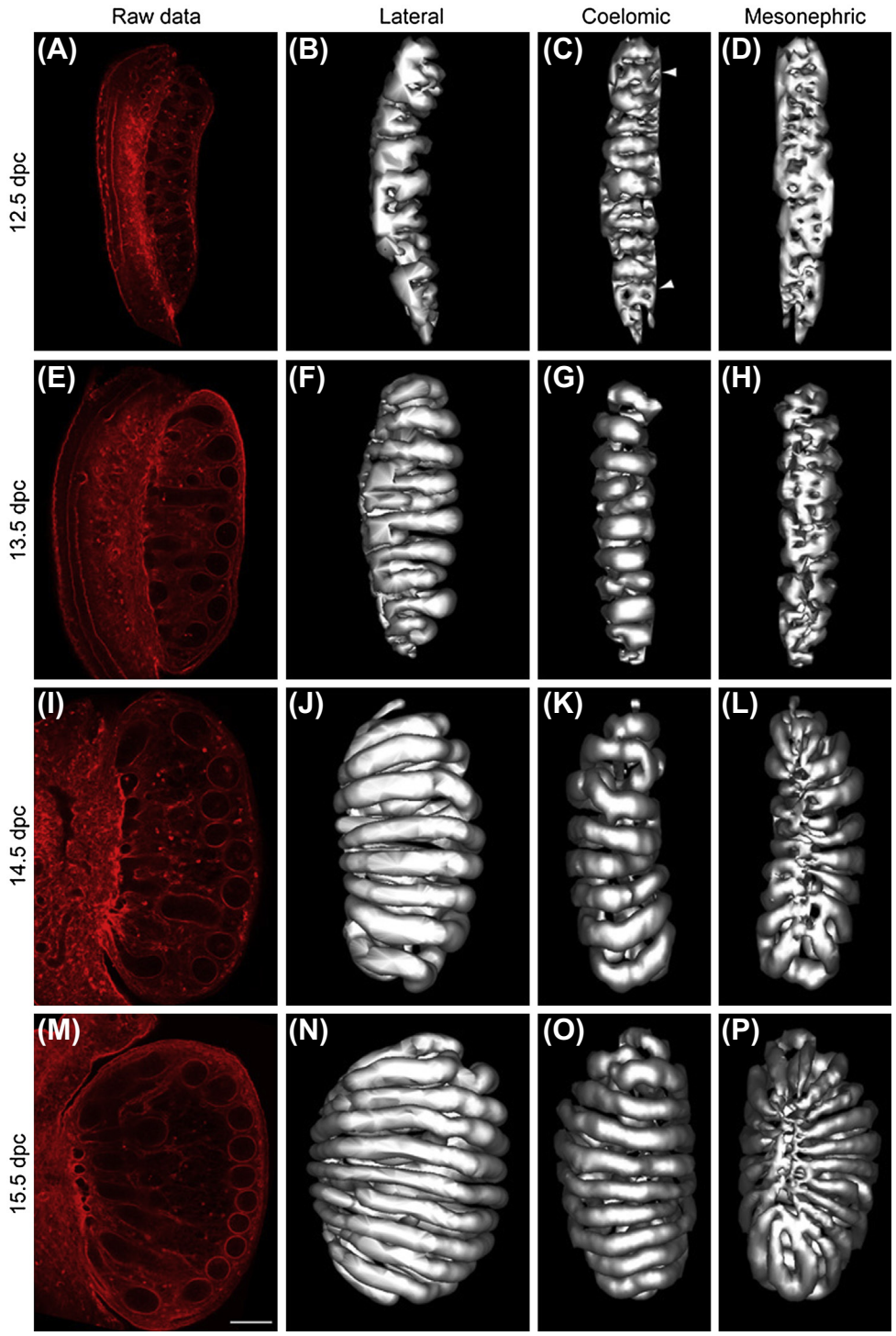

FIGURE 14.2 Modeling of testicular cord development in the mouse. This figure is taken from Ref. 9 and shows lateral (B, F, J, N), coelomic (C, G, K, O), and mesonephric (D, H, L, P) views of models generated from testis cord outlines prepared from whole mount testis. The left panel in each row (A, E, I, M) is a representative image from the data set used to compile the models. The results show that cords initially form as a network of irregular cell clusters that are subsequently remodeled to form regular parallel loops, joined by a flattened plexus at the mesonephric side. Branched, fused, and internalized cords are commonly observed. Parts A–D, E–H, I–L and M–P show models of testicular development at 12.5, 13.5, 14.5 and 15.5 dpc, respectively. Scale bar, 100 μm. *Source: Modified from Ref. 9.*

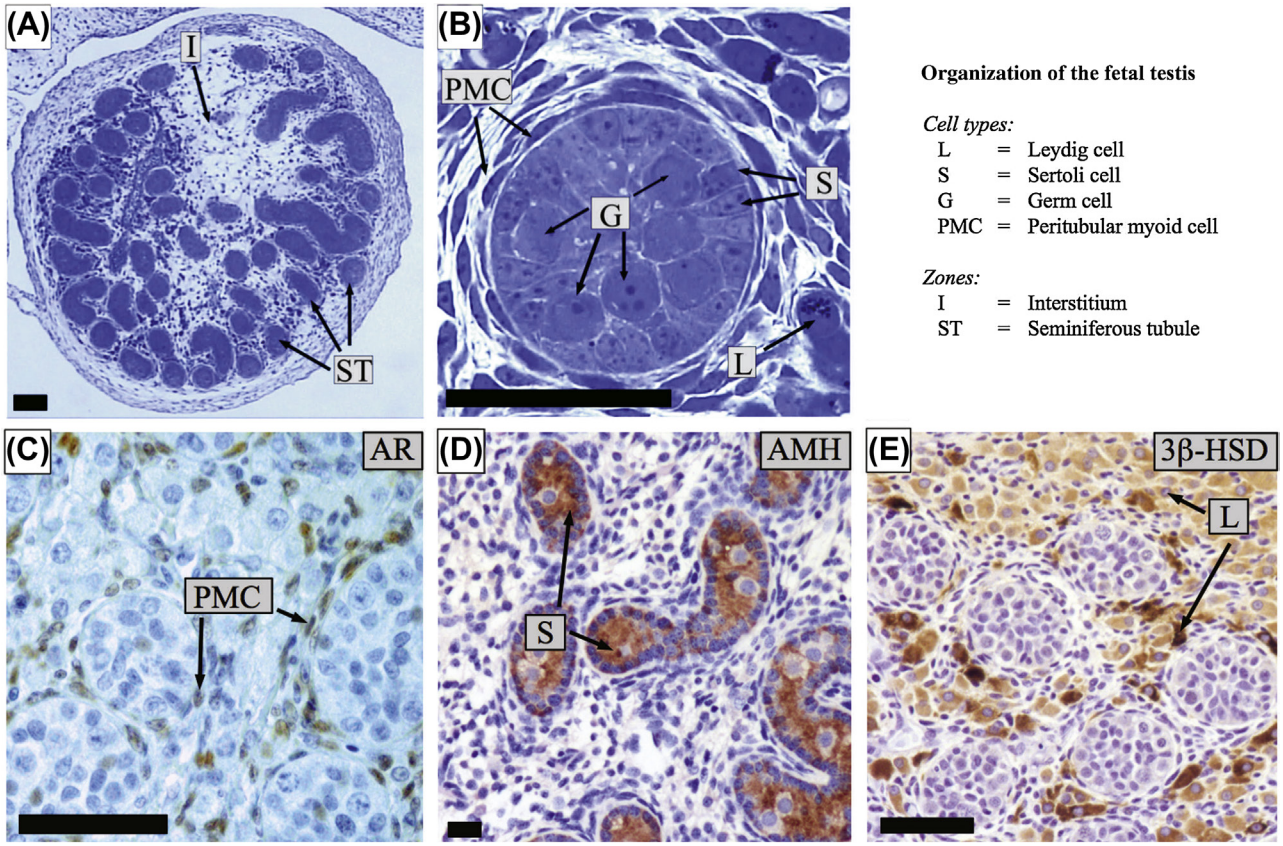

FIGURE 14.3 Histology of the fetal testis. (A) Fetal mouse: low magnification of a semi-thin section showing seminiferous tubules and interstitial tissue (I). (B) Fetal mouse: higher magnification of (A) showing gonocytes (G) in the central part of the sex cord with the Sertoli cells (S) around the periphery. The peritubular myoid cells (PMC) form a concentric layer around the cord, and Leydig cells (L) are present within the interstitium. (C) Fetal human: immunohistochemically labeled for the androgen receptor (AR), which is clearly expressed in PMC and in some interstitial cells. (D) Fetal sheep: immunohistochemically labeled for anti-Mullerian hormone (AMH), which is strongly expressed in fetal Sertoli cells. (E) Fetal human: immunohistochemically labeled for 3β-hydroxysteroid dehydrogenase (HSD3B), which is localized exclusively in the Leydig cells. In all photomicrographs, the bar represents 50 μm. *Source: Reproduced from Ref. 11. © Society for Reproduction and Fertility (2011). Reproduced by permission.*

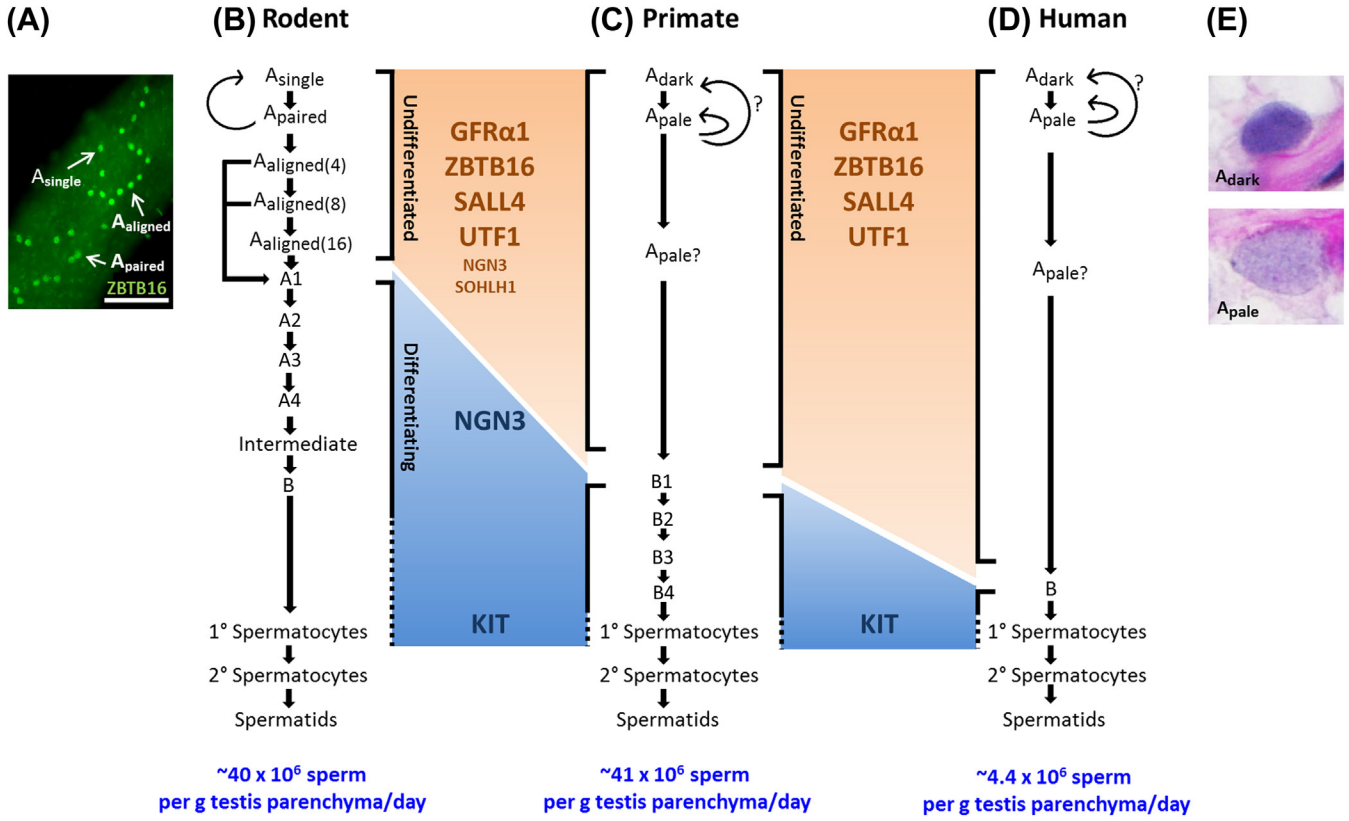

FIGURE 15.2 Current model of rodent, nonhuman primate, and human spermatogenesis. (A) Whole-mount immunohistochemistry staining for ZBTB16 in adult mouse seminiferous tubules. ZBTB16^{+} spermatogonia are identified as A_{single}, A_{paired} or $A_{aligned}$. Scale bar = 100 μm. (B) Rodent undifferentiated spermatogonia, including the SSC pool, is comprised of A_{single} and some A_{paired} spermatogonia and based on whole-mount staining analysis their phenotype is of GFRα1^{+}, ZBTB16^{+}, SALL4^{+}, UTF1^{+}, NGN3$^{+/-}$, and KIT^{-}. Transit amplifying progenitors include some A_{paired} spermatogonia and $A_{aligned}$ spermatogonia (chains of 4–16 cells), with a phenotype of GFRα1^{+}, ZBTB16^{+}, SALL4^{+}, UTF1^{+}, NGN3$^{+/-}$, and KIT$^{+/-}$. The differentiating spermatogonia that are made up of A1–A4, intermediate, and B spermatognia have a phenotype of GFRα1^{-}, ZBTB16^{-}, SALL4^{-}, UTF1^{-}, NGN3$^{+/-}$, and KIT^{+}. (C and D) In nonhuman primate and human testis, the undifferentiated spermatogonia are the type A spermatogonia that are designated A_{dark} and A_{pale} based on nuclear staining intensity with hematoxylin. The B spermatogonia are considered to be the differentiating spermatogonia and in nonhuman primates; they go through four divisions before producing primary spermatocytes, whereas in human there is only one division of B spermatogonia. (E) Sections of human testis stained using periodic acid-Shiff method and counterstained with hematoxylin to show nuclear morphology. ZBTB16, zinc finger and BTB domain containing 16; GFRα1, GDNF family receptor alpha-1; UTF1, undifferentiated embryonic cell transcription factor 1; SALL4, Sal-like 4; NGN3, neurogenin 3; SOHLH1, spermatogenesis and oogenesis specific helix-loop-helix 1. *Adapted from Ref. 75.*

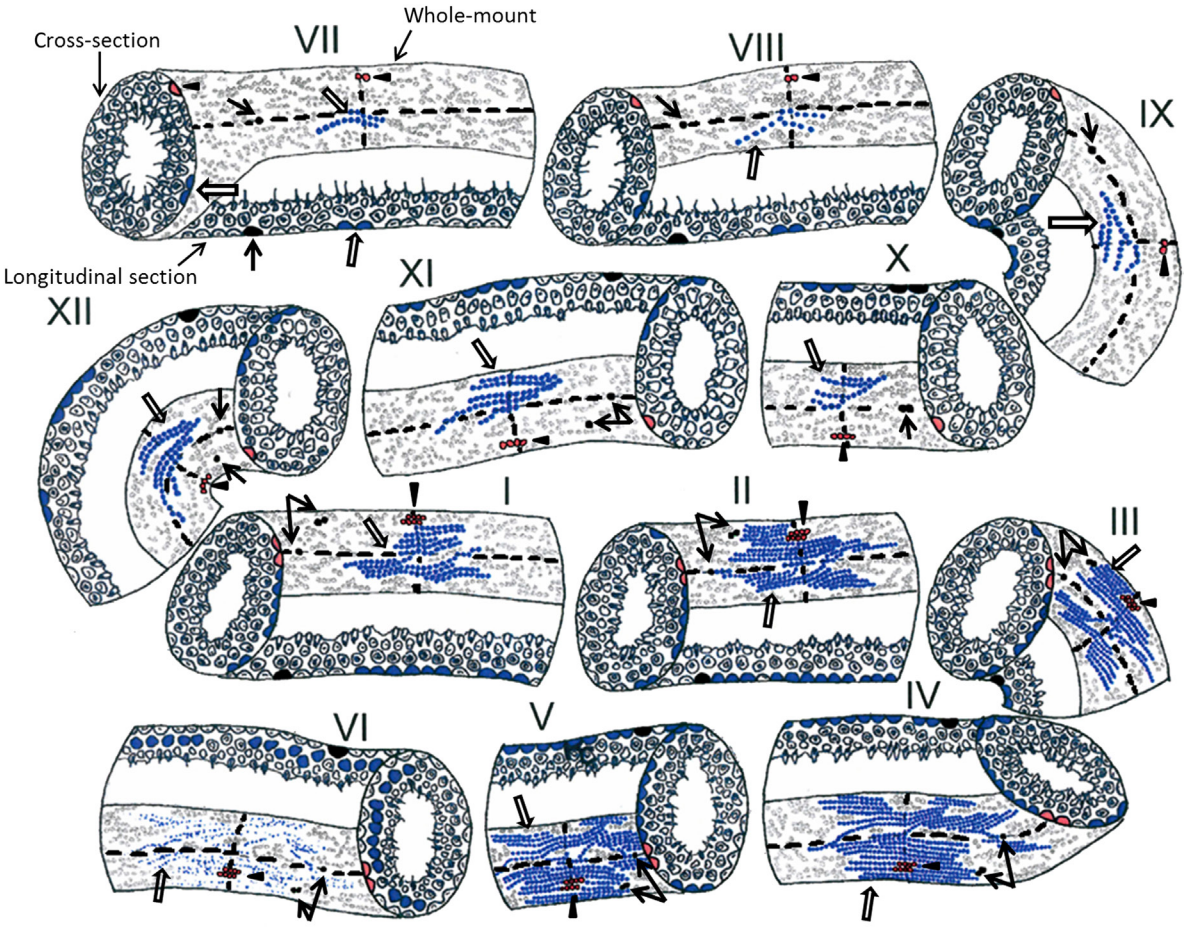

FIGURE 15.3 Schematic of mouse spermatogenic clone development by stage. The seminiferous epithelial cycle of the mouse contains 12 stages (I-XII). Each stage is shown in cross-sectional, longitudinal, and whole-mount perspectives (labeled in stage VII). Three putative spermatogonial clones are highlighted in blue (open arrow), red (arrowhead), and black (black arrow). These clones are only representative examples and do not imply A_{single} spermatogonia divide only in stages X and I. A_{single} and A_{paired} spermatogonia are present in all stages of the seminiferous epithelium and tend to divide more frequently in early and late stages than middle stages. The dotted lines in the whole-mount perspective indicate planes of the cross-section and longitudinal section views. Cells that lie on the focal plane in the whole mount appear in the corresponding cross-section and longitudinal section views; this is indicated by matching color or arrows (arrows shown for stage VII only). For example, in stage VII, the red cell (arrowhead) is in the vertical line and therefore appears in the cross-sectional view. A black cell (black arrow) is in the horizontal line, so it is observed in the longitudinal section view. The development of three putative clones (blue/open arrow, red/arrowhead and black/black arrow) through one cycle of the seminiferous epithelium is shown. Stage VII: $A_{al\text{-}16}$ (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage VIII: A1 (clone of 16) (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage IX: A2 (clone of 32) (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage X: A2 (clone of 32) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{pair} (black/black arrow); stage XI: A3 (clone of 64) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{single} (x2) (black/black arrow); stage XII: A3 (clone of 64) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{single} (x2) (black/black arrow); stage I: A4 (clone of 128) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage II: intermediate spermatogonia (clone of 256) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage III: intermediate spermatogonia (clone of 256) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage IV: Type B spermatogonia (clone of 512) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage V: Type B spermatogonia (clone of 512) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage VI: primary spermatocytes (lifting off the basement membrane) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow). *Reproduced with permission from Ref. 28.*

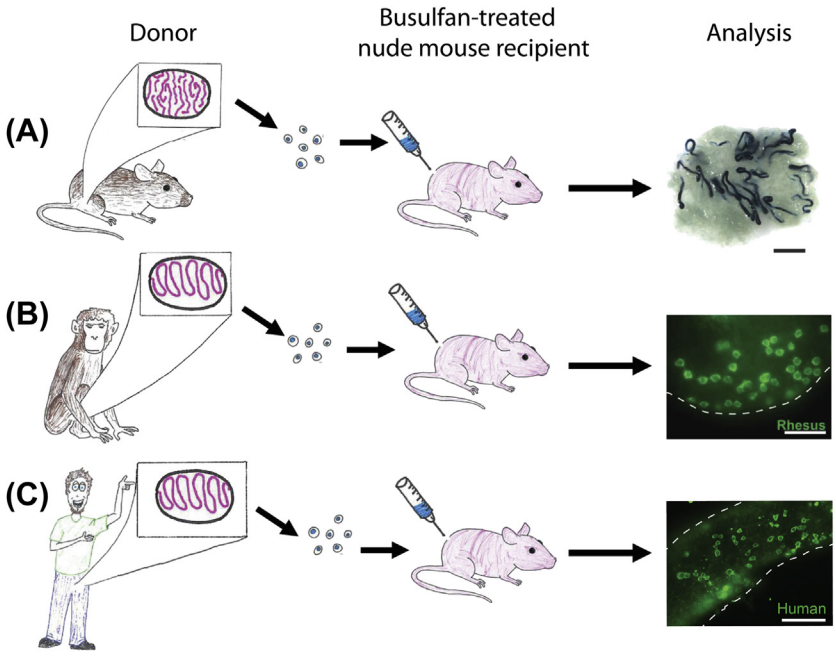

FIGURE 15.6 Spermatogonial stem cell (SSC) transplantation assay. The only way to definitively identify SSCs is to analyze SSC function. (A) In this case, a testicular cell suspension is produced from a *lacZ* donor mouse. The cells are injected into the testis of an infertile recipient mouse and the results are analyzed two to three months after transplantation for *lacZ* expressing donor spermatogenesis (dark colonies). Scale bar = 2 μm. (B, C) Xenotransplantation of nonhuman primate (B) and human (C) testicular cells into busulfan treated nude mouse can be used as an assay to identify SSCs by their ability to initiate the early steps of spermatogenesis in the mouse testis. The colonies of spermatogonia are identified by staining with anti-primate antibody that only recognizes primate cells. The colonies of SSCs are identified by the following criteria—they are located on the basement membrane of the seminiferous tubules, they have four or more cells, and the cells are within 100 μm of each other. Scale bars = 100 μm. In the B and C analysis panel, broken lines indicate the borders of seminiferous tubules.

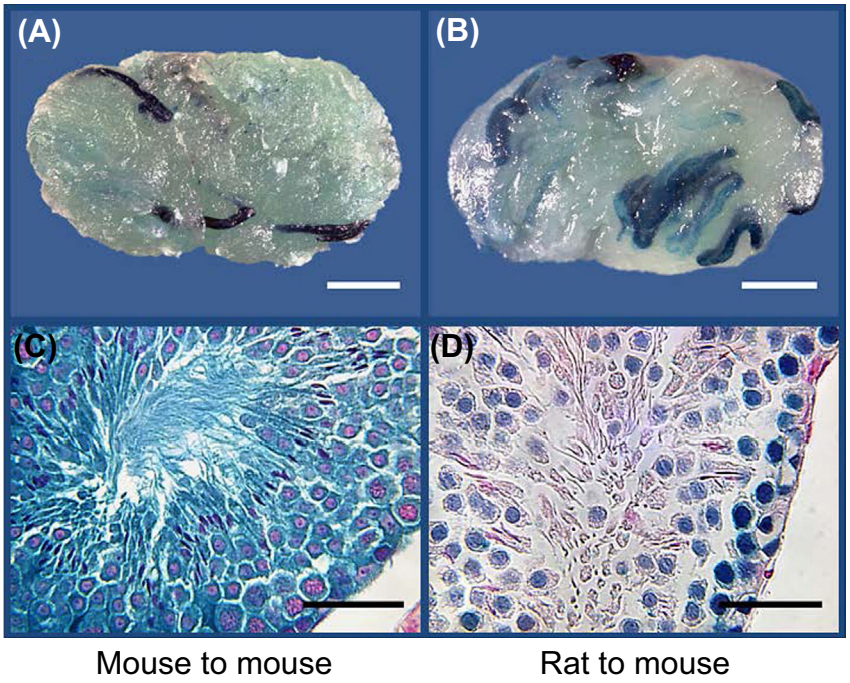

FIGURE 15.7 Macroscopic and histologic appearance of recipient testes transplanted with mouse and rat adult donor testis cells expressing *lacZ*. NCr nude (spontaneous mutant T cell deficient mice) recipient mouse testes were stained with X-gal 3 months after transplantation to visualize mouse (A and C) and rat (B and D) donor-derived spermatogenic colonies. Both mouse and rat donor testis cells generated complete spermatogenesis, characterized by multiple germ cell layers and mature spermatozoa, in recipient testes. Note that the lacZ stain is localized in the cytoplasm of ROSA26 mouse testis cells (C) and the nucleus of MT-lacZ rat testis cells (D). Nuclear fast red counterstain (C and D). Bar = 1 mm (A and B), 40 μm (C and D). *Reproduced with permission from Ref. 134.*

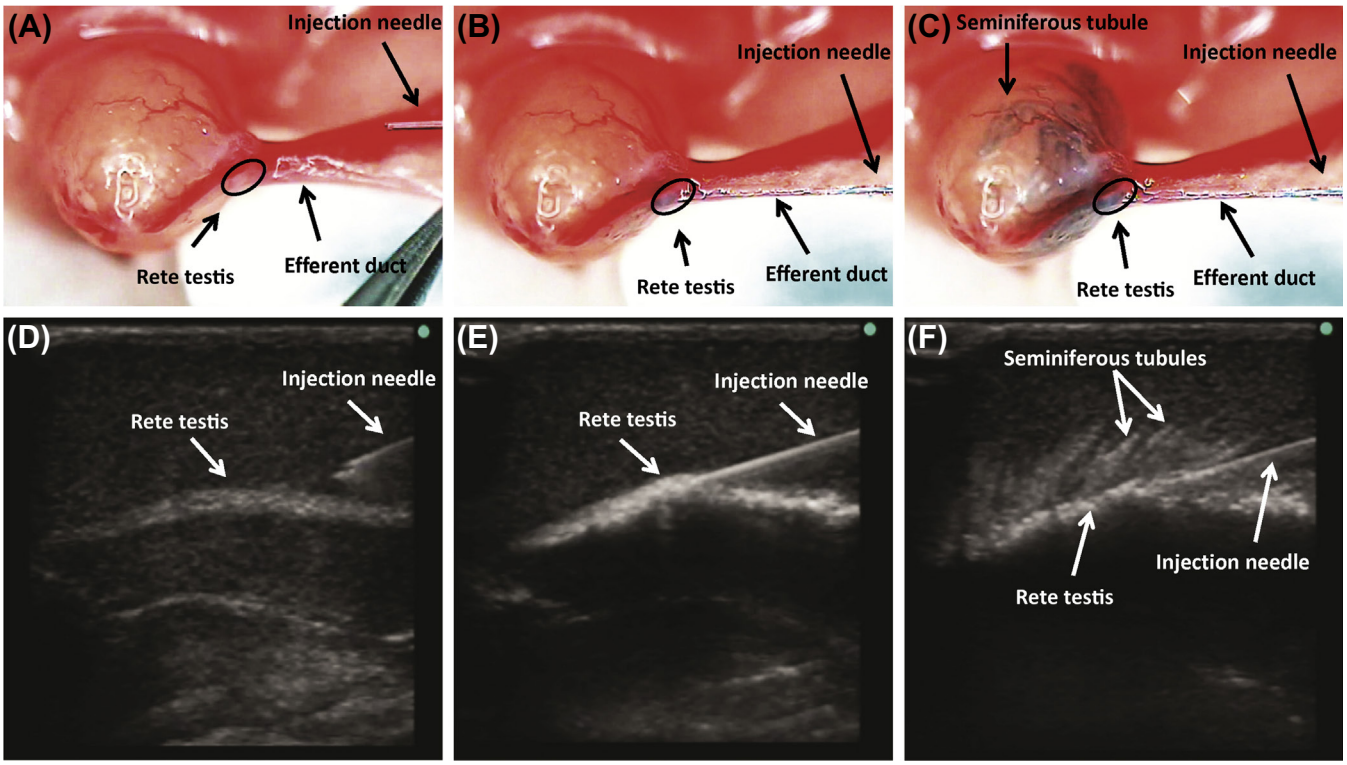

FIGURE 15.8 Testicular cell transplantation. (A–C) In rodents, the testicular cells are injected via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules. (C) Trypan blue is injected with the testicular cells to visualize the filling of the seminiferous tubules. (D–F) Testis anatomy in large animals is different than rodents, with the rete testis being more centrally localized and therefore more difficult to visualize and access. Therefore, ultrasound is used to guide injections. (D) Rete testis (echo-dense structure) is visible on ultrasound. The injection needle is inserted under ultrasound guidance through the scrotal skin into the rete testis space, which is continuous with the seminiferous tubules. (E) Positive pressure is applied to the needle, so the cells are slowly injected into the rete testis and seminiferous tubules. (F) The filling of the seminiferous tubules is observed using microbubbles.

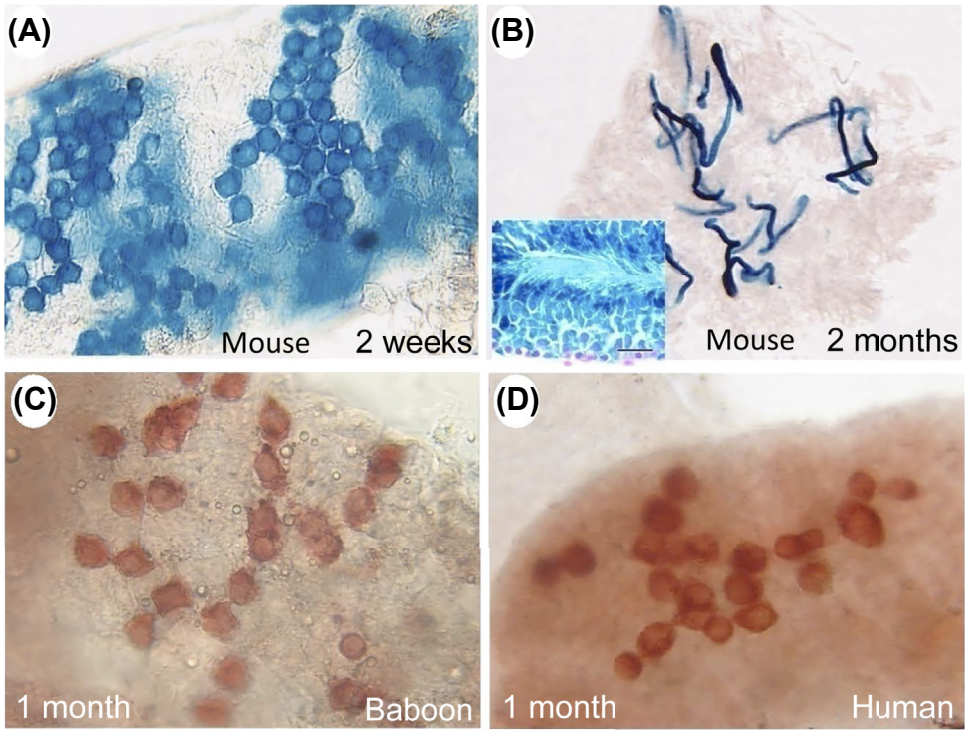

FIGURE 15.9 Colonization of recipient testis. (A) Two weeks after transplantation of transgenic mouse donor cells (stained blue) into a recipient mouse seminiferous tubules, small groups of cells can be seen at low magnification. (B) Two months after the transplantation of mouse donor cells, the blue areas are dark in the center, indicating multiple layers of germ cells and development of spermatogenesis. (B inset) Cross-section of a transplanted mouse seminiferous tubule 2 months after transplant. Scale bar = 30 μm. (C and D) Detection of baboon and human germ cells transplanted into the seminiferous tubules of recipient mouse, using an antibody raised against baboon testis cells. One month after the transplant, clusters of baboon (C) and human (D) germ cells are located on the basement membrane of mouse tubules and exhibit characteristics of undifferentiated spermatogonia. The clusters are similar to the ones seen in mouse 2 weeks after the transplant. *Modified with permission from Refs 104 and 149.*

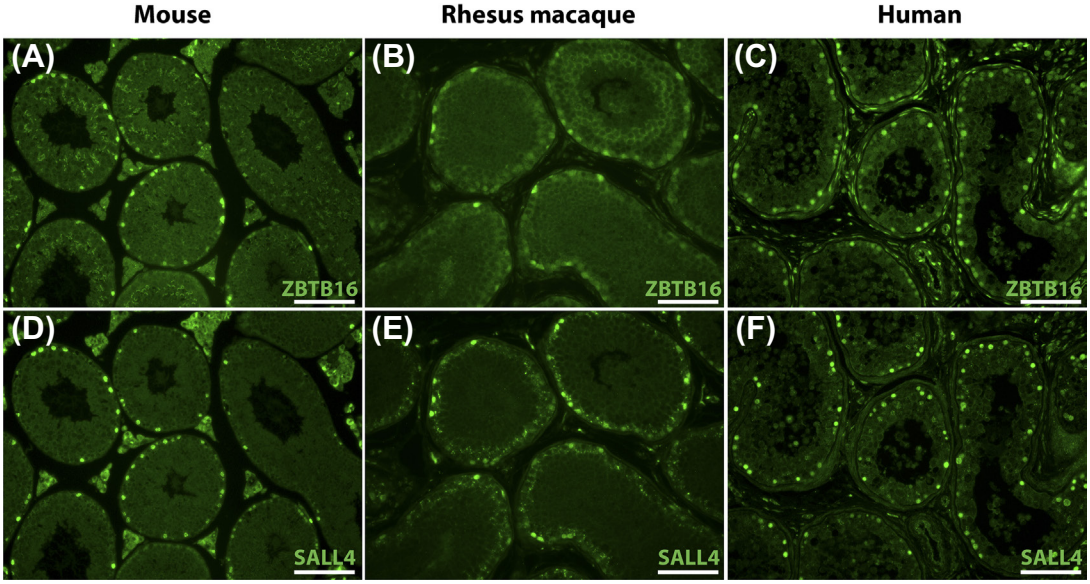

FIGURE 15.10 Conservation of stem and progenitor markers ZBTB16 and SALL4 from mouse to human. ZBTB16 (A–C) and SALL4 (D–F) staining in cross-sections of seminiferous tubules from mouse, rhesus macaque, and human testes shows expression of these transcription factors on the basement membrane of the tubule. Both ZBTB16 and SALL4 are conserved markers from mouse to rhesus macaque to human. Scale bar = 100 μm. ZBTB16, zinc finger, and BTB domain containing 16; SALL4, Sal-like 4.

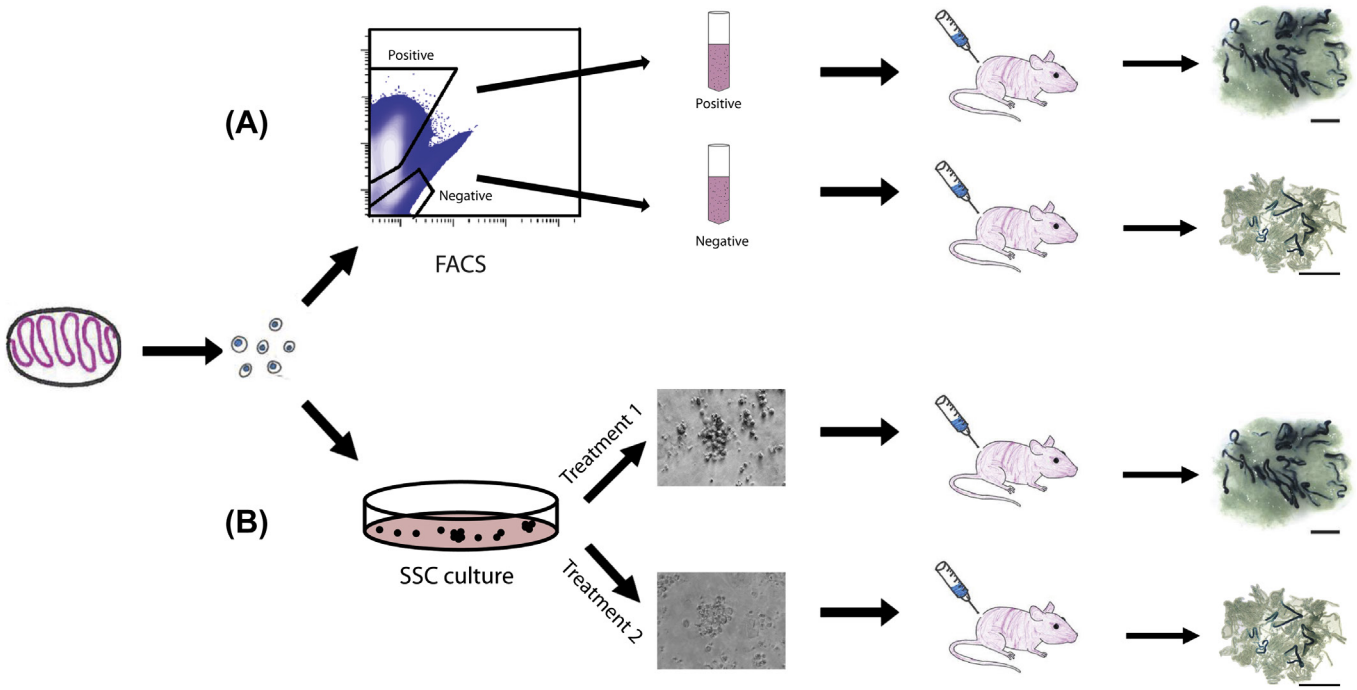

FIGURE 15.11 Experimental designs to study seprmatogonial stem cells. SSC transplantation is used to analyze the effectivness of (A) fluorecsence-activated cell sorting (FACS) for a cell-surface protein or (B) a treatment of SSC culture. (A) FACS is used to fractionate the testicular cell population stained with an antibody against a cell-surface protein, based on marker$^+$ and marker$^-$ status. The sorted fractions can then be transplanted into infertile nude mice to determine which fraction contains the SSCs. (B) SSC cultures are used to identify factors important for SSC self-renewal and maintenance or for SSC differentiation. For example, GDNF can be removed from the culture media to determine whether or not it has an effect on SSC self-renewal and/or maintenance. The cells cultured with or without GDNF are then transplanted into testis of infertile recipient mice and, 2 months later, the testis are analyzed for colony formation. FACS, fluorescence activated cell sorting; SSC, spermatogonial stem cell; GDNF, glial cell-derived neurotrophic factor.

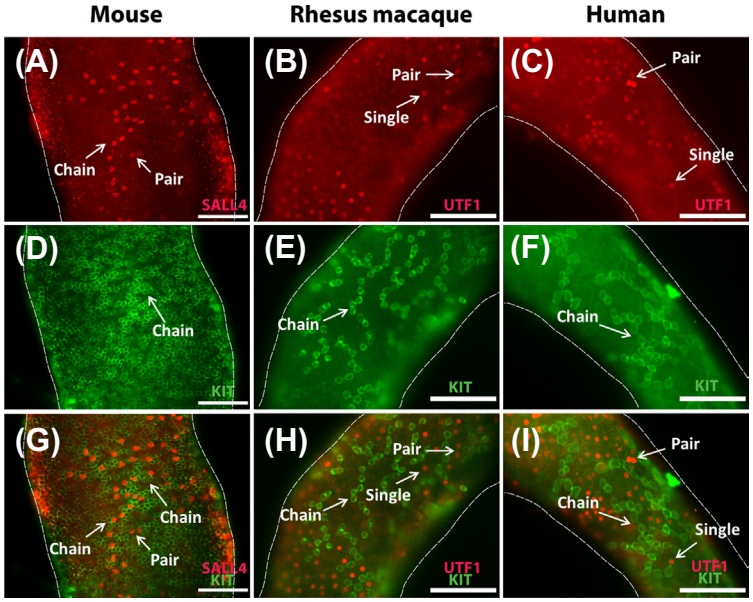

FIGURE 15.12 Whole-mount immunohistochemistry of seminiferous tubules from mouse, rhesus macaque, and human testes. Whole-mount immunofluorescence analysis of the undifferentiated spermatogonia marker, SALL4 (A), differentiating spermatogonia marker, KIT (D) and co-staining of SALL4 and KIT (G) in mouse. Whole-mount immunofluorescence analysis of undifferentiated spermatogonia marker UTF1 (B, C), differentiating spermatogonia marker KIT (E, F), and co-staining of UTF1 and KIT (H, I) in the rhesus macaque (B, E, H) and human (C, F, I). Arrows indicate spermatogonia arranged as single cells, pairs, and larger chains for each species. Scale bar = 100 μm. SALL4, Sal-like 4; UTF1, undifferentiated embryonic cell transcription factor 1.

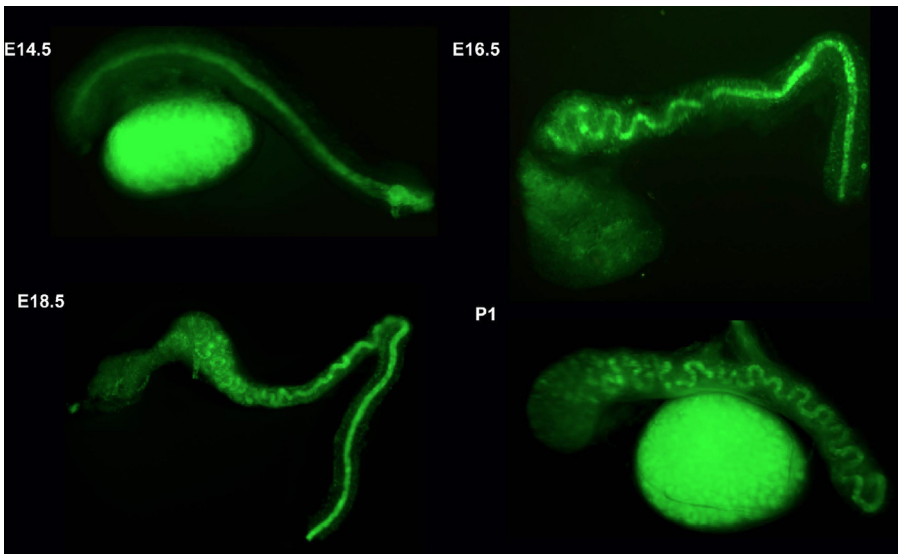

FIGURE 17.2 Wolffian ducts collected from hoxb7-gfp mice at different developmental stages, embryonic (E) days 14.5, 16.5, 18.5 and postnatal (P) day 1. Note how the coiling proceeds from proximal to distal with three-dimensional coiling beginning around 18.5. *Source: Image from Ref. 60.*

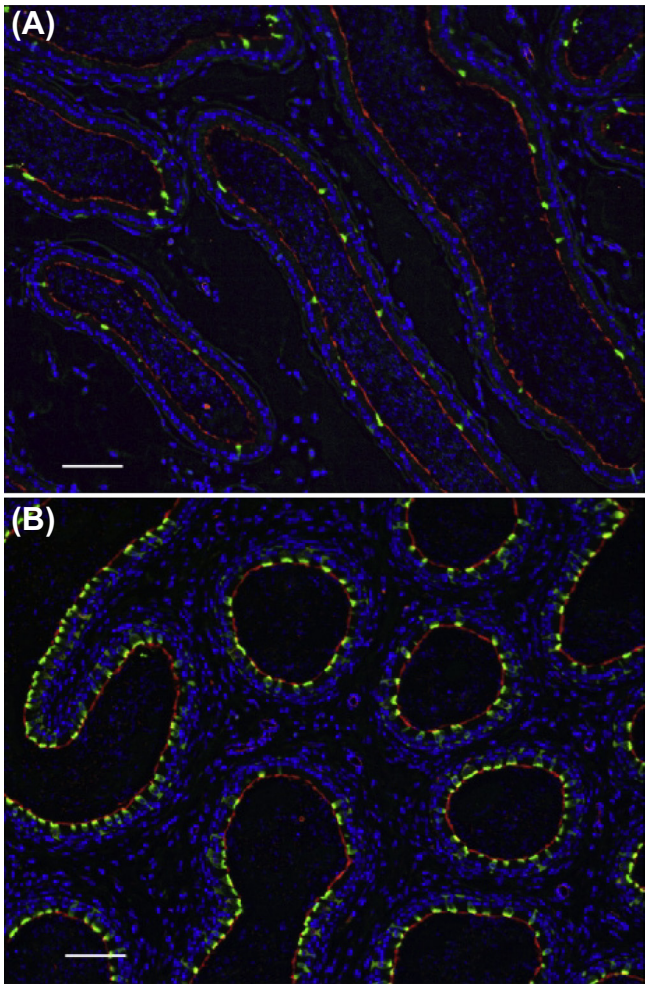

FIGURE 17.8 Relative numbers of clear cells in the rat caput (A) versus cauda (B) epididymidis. Rat epididymis was stained for the V-ATPase B1 subunit (green) to label clear cells, and NHERF1 (red). Nuclei and spermatozoa were stained with DAPI (blue). NHERF1 is located in the apical membrane of both principal cells and clear cells. Clear cells positive for the subunit B-1 or V-ATPase are much more numerous in the cauda (B) than in the caput (A) epididymidis. Scale bars, 50 μm. *Source: Reproduced with permission from Ref. 164.*

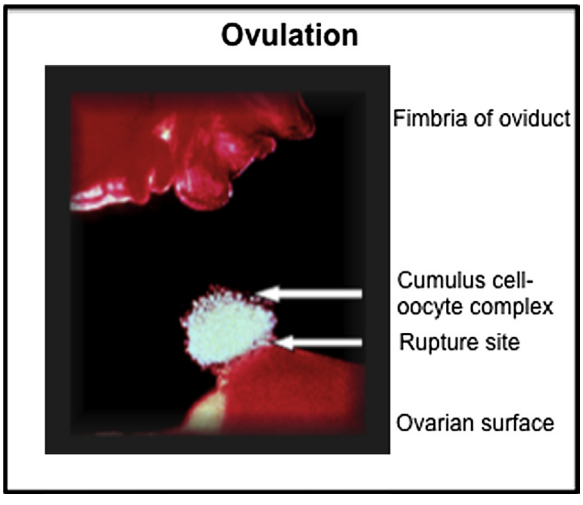

FIGURE 22.1 Moment of rupture of a rabbit follicle. Ovulation of a fertilizable oocyte involves disintegration of the ovarian surface epithelium and extrusion of the expanded cumulus cell–oocyte complex (COC) through the ovulation pore. This is followed by retrieval of the COC by the ciliated cells of the oviduct and its subsequent transport down the oviduct where fertilization occurs. (See text.) *Source: Ref. 3.*

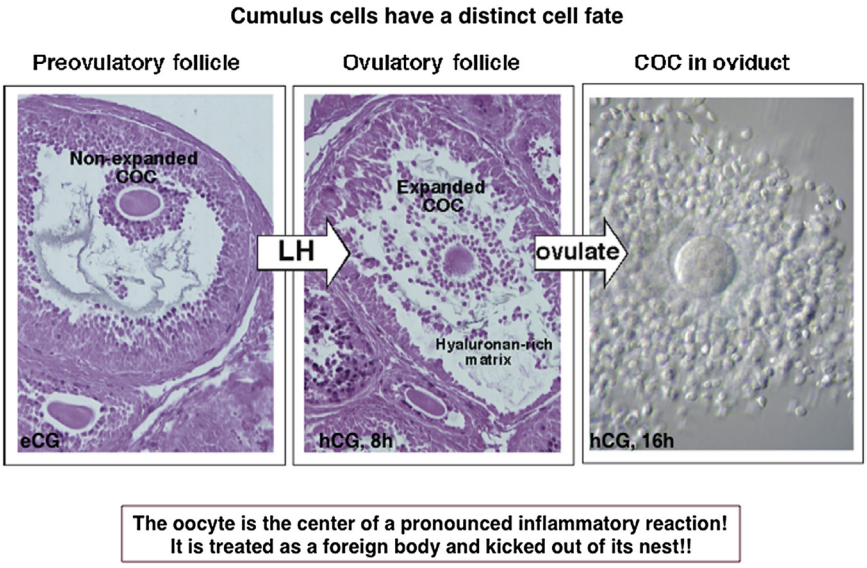

FIGURE 22.3 **The LH surge induces expansion of the cumulus cell-oocyte complex (COC).** Expansion is dependent on the biosynthesis of long polymers of hyaluronan by HAS2. The hyaluronan polymers are stabilized by specific hyaluronan-binding factors including the proteoglycan versican and inflammatory-related factors such as TNFAIP6, IαI, and PTX3. COC expansion is obligatory for release of the enclosed oocyte from the surface of the ovary into the oviduct. (See text.)

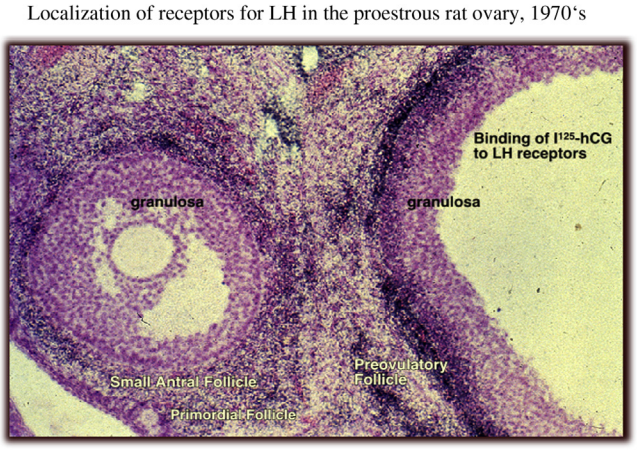

FIGURE 22.5 **Induction of LH receptors (LHR; LHCGR) in granulosa cells is the key distinguishing hallmark of preovulatory follicles.** The presence of LHR in granulosa cells of preovulatory follicles permits them (but not small follicles lacking LHR in granulosa cells) to respond to the LH surge and initiate the molecular programs required for ovulation, COC expansion, resumption of meiosis, and luteinization. This image shows the uptake of radiolabeled hCG (LH) into the theca cells and mural granulosa cells of a preovulatory follicle (right) but only in theca cells of small follicles (left). Primordial follicles do not exhibit detectable uptake of the hormone. Uptake reflects the presence of receptors.

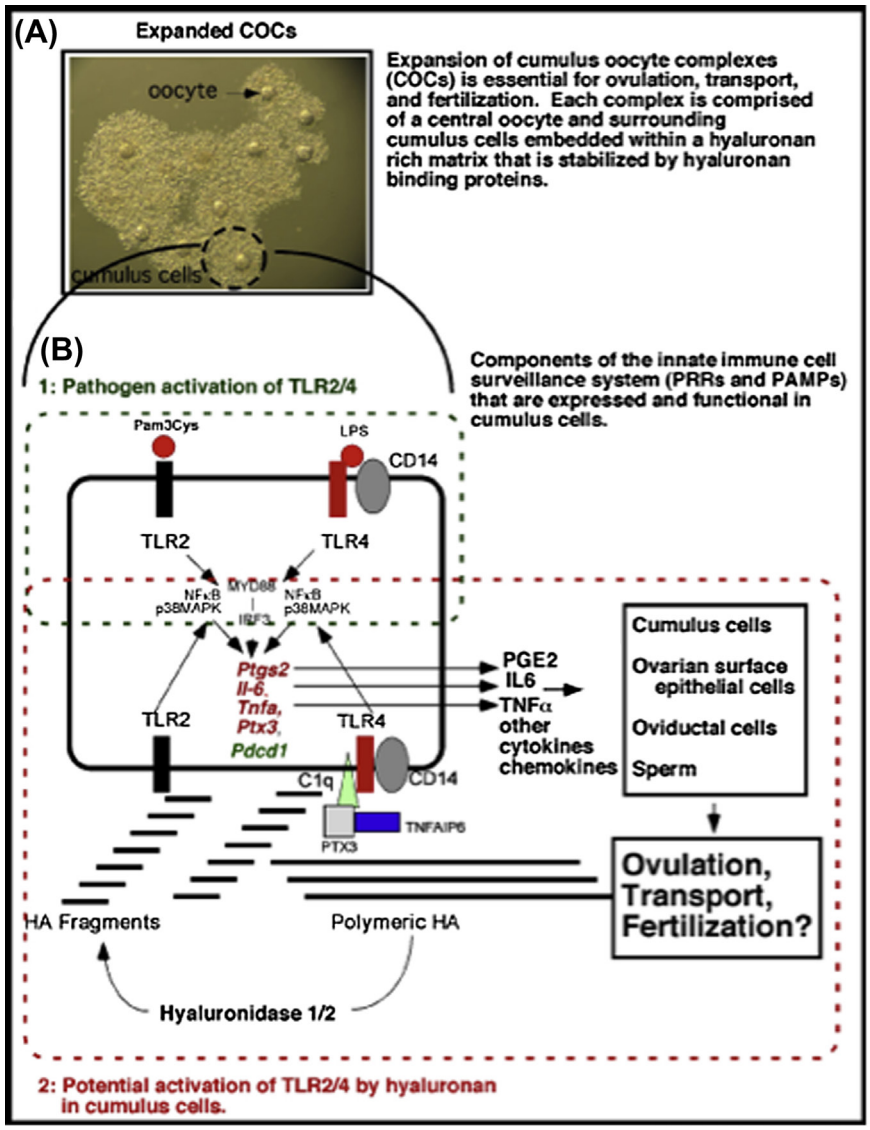

FIGURE 22.12 Schematic of TLR pathway activation and functions in cumulus cells during COC expansion, ovulation, transport and fertilization. (A) In response to the ovulatory surge of LH, cumulus cell–oocyte complexes (COCs) undergo expansion. This process requires the synthesis of a hyaluronan (HA)-rich matrix and factors that bind HA to stabilize the matrix. This process is critical for ovulation. (B) Cumulus cells express members of the Toll-like receptor (TLR) superfamily and can respond to specific ligands (1: pathogen-derived or 2: matrix-derived), leading to the induction of inflammation- and innate immune-related genes. These include: *Ptgs2, Il6, Tnfaip6, Tnfa*, and *Pdcd1*. Prostaglandins (PGE2) synthesized by PTGS2, IL6, and TNFα, as well as other cytokines and chemokines, are released from cumulus cells and can impact the function not only of the cumulus cells but also oviductal cells during transport and sperm during fertilization. The degradation of polymeric HA by hyaluronidases is presumed to lead to the generation of HA fragments that activate TLR2 and TLR4. *Source: Ref.160.*

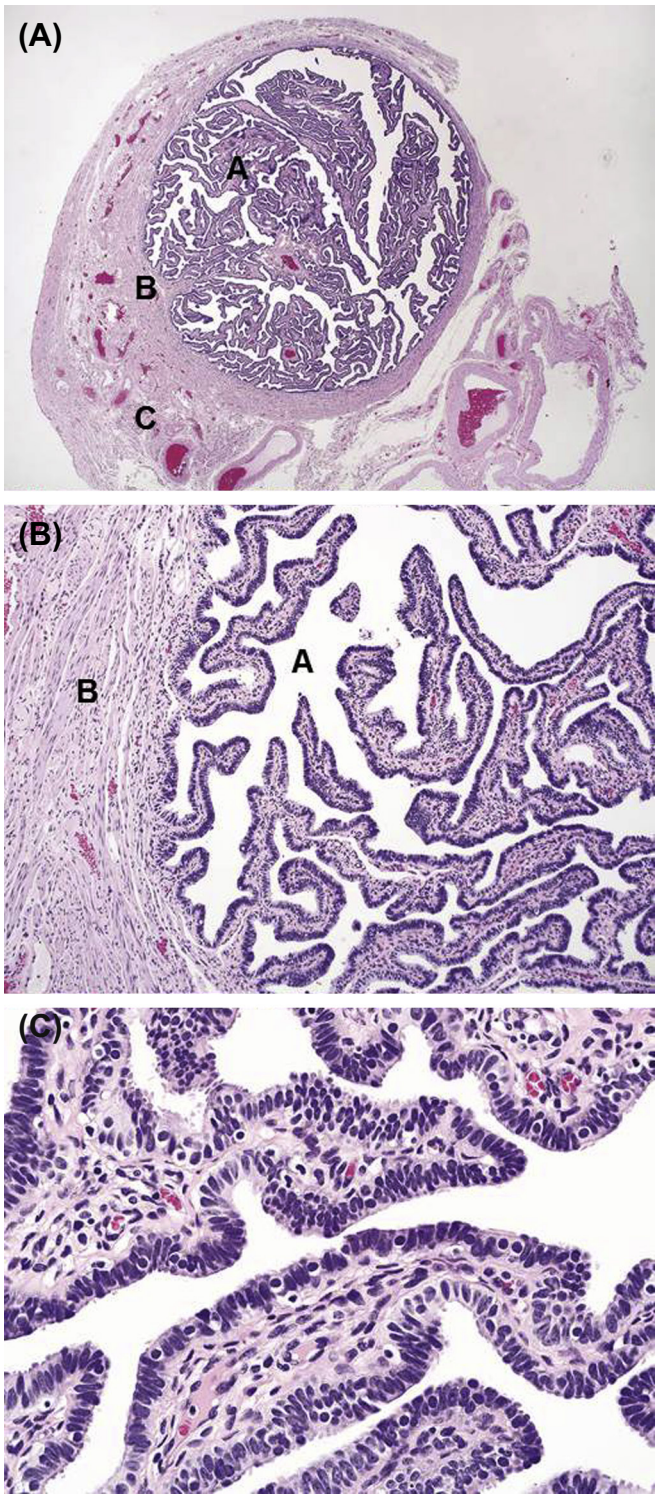

FIGURE 24.2 Cross-section of human fallopian tube, ampulla. Inner mucosa (A) is arranged in longitudinal folds within the middle muscularis (B) and outer serosa (C). The mucosa is lined by a single layer of epithelium (lower panel) composed of ciliated and secretory (nonciliated) cell types. Hematoxylin and eosin (H & E) stain, magnification 20–400×. *Source: Images courtesy of Ramya Massand and Jason Moss.*

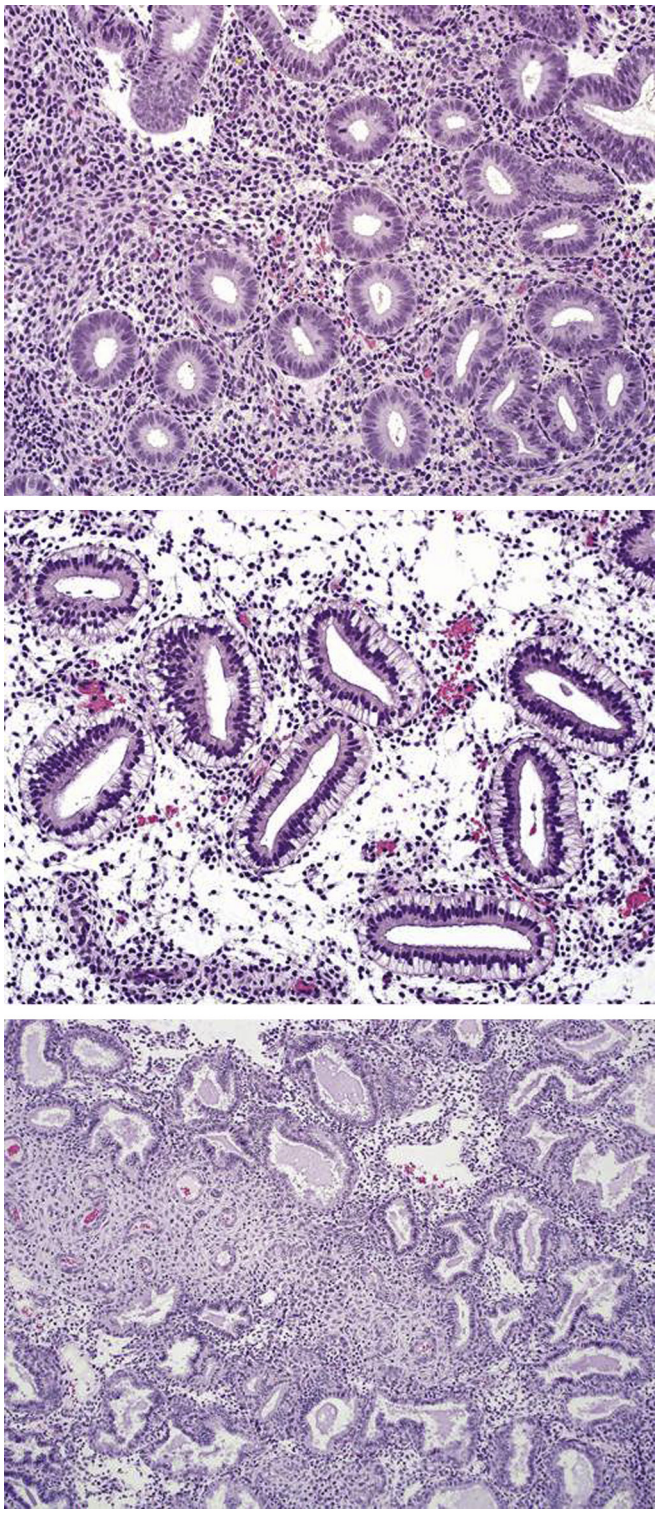

FIGURE 24.4 Human endometrium from proliferative (upper), early-secretory (middle), and late-secretory phases (lower). Note the wide spacing between simple-appearing glands (proliferative), apically displaced nuclei in the glandular epithelium due to glycogen accumulation (middle), and tortuous glands with secretions and a decidualized stroma with abundant pale cytoplasm (lower). H&E stain, magnification 100–200× (Images courtesy of Ramya Massand and Jason Moss).

Color Plates

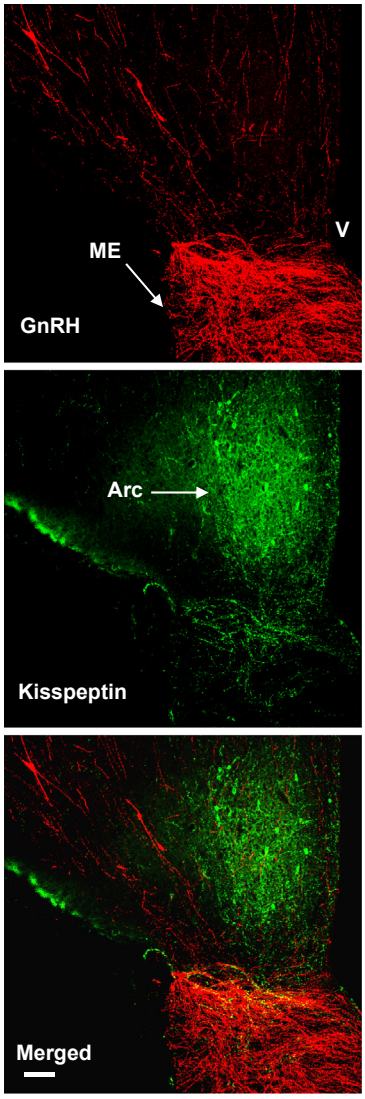

FIGURE 28.3 **A hemi-coronal section through the mediobasal hypothalamus of a castrated rhesus monkey at the level of the arcuate nucleus (Arc), the posited site of the gonadotropin-releasing hormone (GnRH) pulse generator.** The section was doubled labeled by immunofluorescence for GnRH and kisspeptin. Top panel, a confocal image of the distribution of GnRH cell bodies and fibers; middle panel, also a confocal image of the same section showing the corresponding distribution of kisspeptin cell bodies and fibers. The lower panel shows the merged image. Castration results in an upregulation of kisspeptin expression that is associated with robust GnRH pulse generator activity. Note that GnRH cell bodies at this level of the hypothalamus are found lateral to the kisspeptin perikarya in Arc: both sets of neurons send projections to the median eminence (ME) where they closely intermingle. The ependymal lining of the third ventricle (V) may be seen on the right-hand side of the section. Scale bar 100 μm. *Source: Rearranged from Ref. 57, with permission, Copyright 2008, The Endocrine Society.*

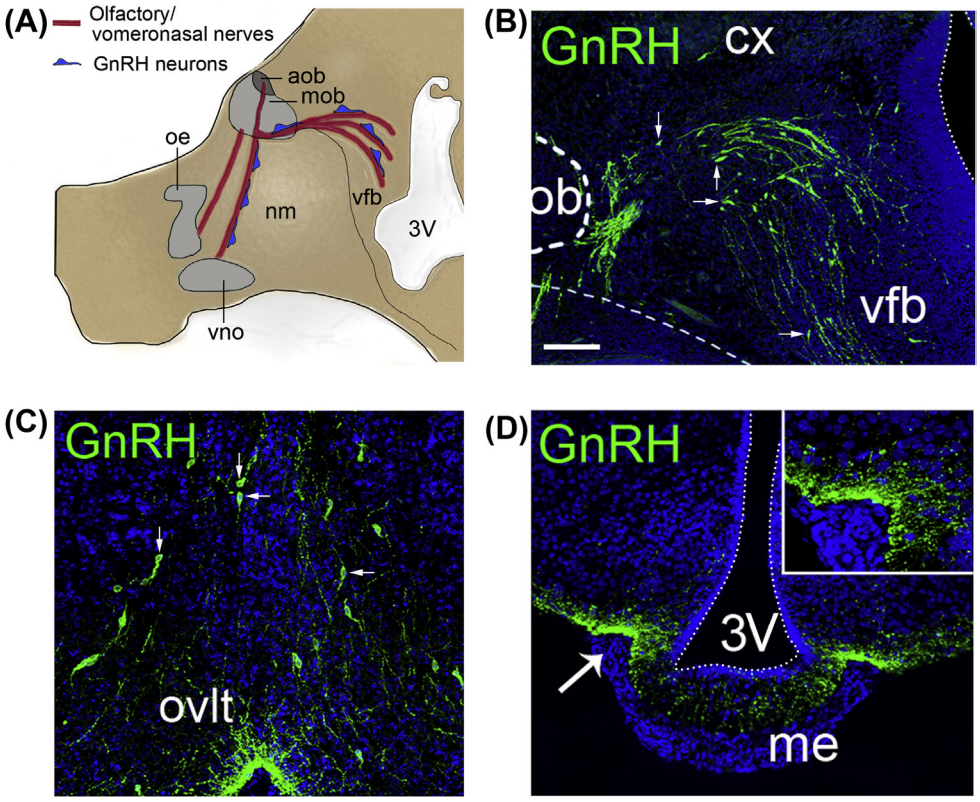

FIGURE 30.1 (A) Schematic representation of the head of a mouse embryo at E14.5, showing the scaffold of vomeronasal/terminal nerve fibers (thick lines) along which GnRH cells (neuron-like shapes) migrate from the nose to the brain. (B) Sagittal section of the rostral and ventral forebrain regions at E14.5, immunolabeled for GnRH. Arrows show GnRH neuronal cell bodies. (C, D) Coronal section of the preoptic region showing GnRH neuroendocrine cells (arrows, C) and their nerve terminals in the median eminence (me, D) in newborn (P0) mice. In D, the inset shows a higher magnification of the area identified by the arrow in the main panel. Abbreviations: oe, olfactory epithelium; vno, vomeronasal organ; nm, frontonasal mesenchyme; mob, main olfactory bulb; aob, accessory olfactory bulb; vfb, ventral forebrain; cx, cerebral cortex; ovlt, organum vasculosum of the lamina terminalis; 3V, third ventricle. Scale bar: 100 μm (50 μm in inset).

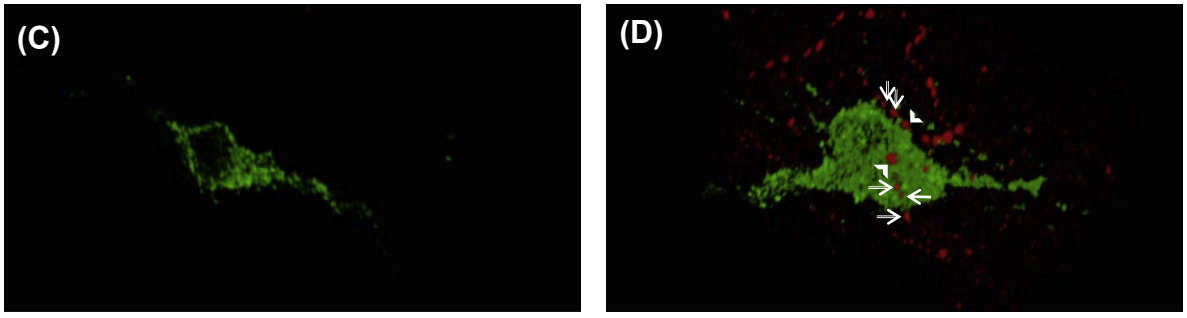

FIGURE 31.8 Changes in LH pulse frequency and kisspeptin during pubertal development. *Panel A*: LH pulse frequency in prepubertal and postpubertal female lambs that were either gonad-intact (Intact) or ovariectomized (OVX). Postpubertal intact lambs were in the early follicular phase. LH pulse frequency was higher in intact postpubertal female lambs than in intact prepubertal female lambs and rose following ovariectomy in the prepubertal, but not postpubertal, lambs. *Panel B*: Kisspeptin-immunopositive cell numbers were greater in postpubertal intact females than prepubertal intact females and increased with ovariectomy in the prepubertal, but not postpubertal, lambs. *Panels C and D*: GnRH neuron from a prepubertal female (panel C) and postpubertal female (panel D). Heavy arrows indicate point of contact between kisspeptin-containing varicosities and the GnRH neuron. Lighter arrows depict kisspeptin-positive nerve fibers. *Panels E and F*: The percentage of GnRH neurons exhibiting at least one kisspeptin close contact (panel E) was greater in intact postpubertal females than intact prepubertal females. This percentage increased with ovariectomy in the prepubertal, but not postpubertal, group. Numbers of contacts per GnRH neuron (panel F) did not differ among groups. *Redrawn from Ref. 72, with permission.*

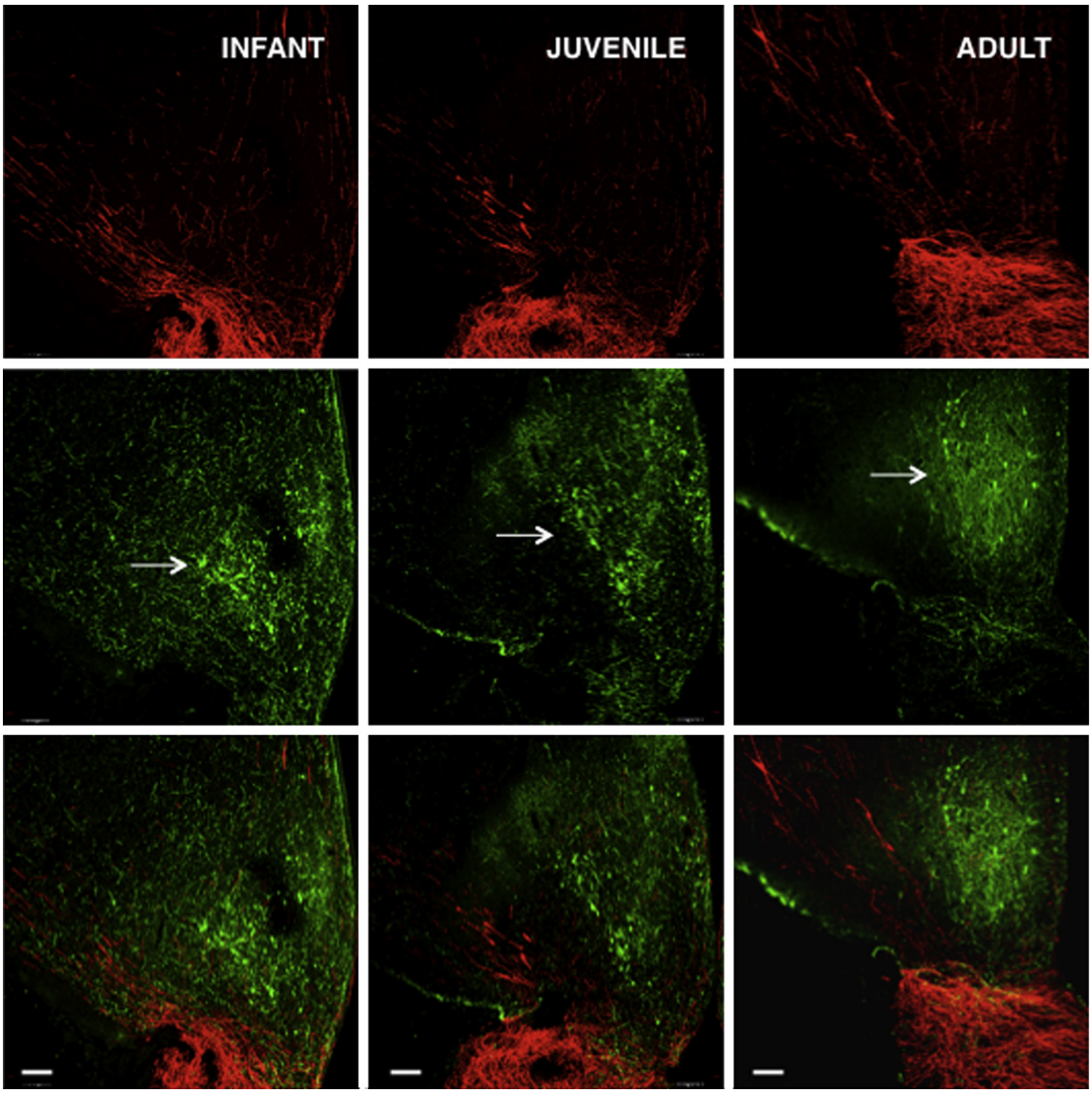

FIGURE 32.6 Confocal images illustrating developmental changes in GnRH (top panels) and kisspeptin (middle panels) neurons (immunopositive perikarya and fibers) in the MBH at the mid-tuberal level during postnatal development in the agonadal male rhesus monkey. The lower panels show the merged images. A hemi-hypothalamic section is shown for an infant (left-hand panels), juvenile (center panels), and adult (right-hand panels) animal; stages of postnatal development corresponding to the on-off-on pattern of GnRH pulse generator activity in primates. Note that kisspeptin immunoactivity is reduced in the juvenile MBH at a time when GnRH pulsatility is arrested but GnRH immunoactivity in the median eminence is maintained during this phase of development. In most cases the ependymal lining of the third ventricle is visible on the right-hand boundary of each hemi-section. *Arrow* indicates kisspeptin neurons in the arcuate nucleus. Scale bar, 100 μm. *Source: Parts of this montage have been reprinted with permission from Refs 170,266.*

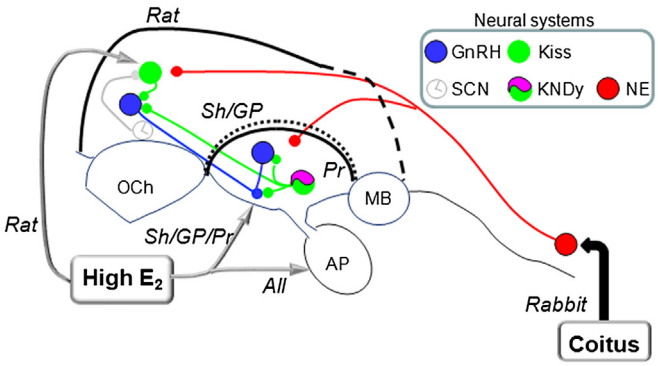

FIGURE 33.8 Schematic of parasagittal section of the hypothalamo–hypophysial unit illustrating the anatomical areas and neural systems participating in the preovulatory GnRH surge and sites of E_2 positive feedback in various species. All structures are required in rabbits; in other species, solid lines depict knife cuts that had no effect on the surge. Dotted lines are cuts that only decreased the amplitude of the LH surge, and the dashed line illustrates that NE input is normally required for the surge in rats but can be compensated for under some circumstances. See the text for more details. AP: anterior pituitary; GP: guinea pig; MB: mammillary body; OCh: optic chiasm; Pr: primate; Sh: sheep.

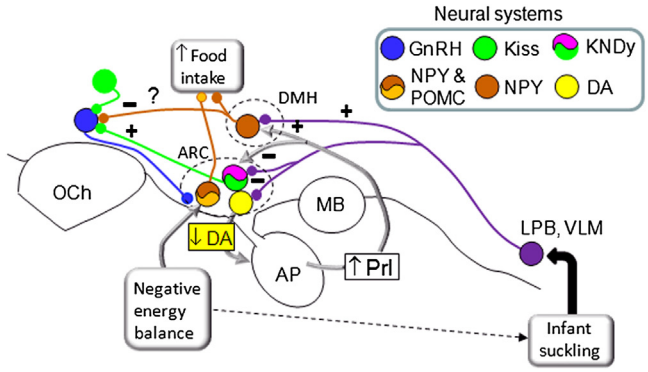

FIGURE 33.12 Neural systems stimulating food intake and inhibiting GnRH secretion during lactation in the rat. The suckling stimulus is transmitted to the hypothalamus via neurons in the lateral parabrachial nucleus (LPB) and ventrolateral medulla (VLM), inhibits DA release from tuberoinfundibular dopaminergic (TIDA) neurons and kisspeptin release from KNDy neurons, and stimulates NPY neural activity in the DMH. The fall in DA allows prolactin secretion to increase, which further inhibits KNDy kisspeptin and stimulates NPY from the DMH. The latter increases food intake, while the former inhibits GnRH secretion. At the same time, the negative energy balance in the mother induced by the demands of the offspring acts via the metabolic homeostatic circuitry in the ARC (increased NPY–AgRP and decreased POMC–CART), which also acts to stimulate food intake and may inhibit GnRH secretion. Note that this circuitry is depicted by a single neural element (NPY and POMC) for simplicity. Similarly, orexogenic neurons in the LHA and anorexogenic CRH neurons in the PVN are not illustrated.

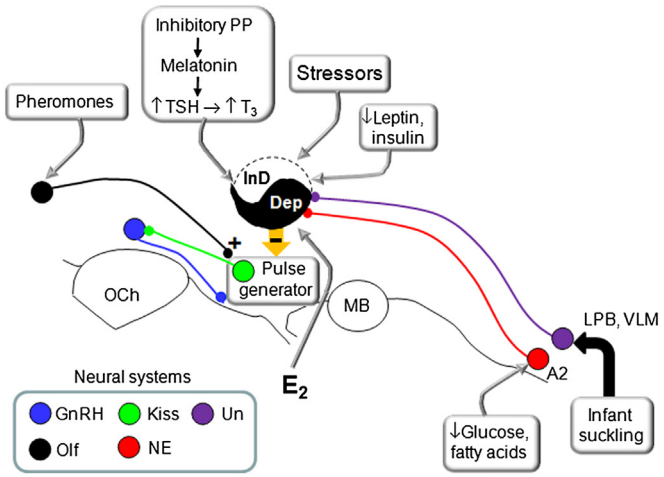

FIGURE 33.13 Schematic of parasagittal section of the hypothalamus illustrating mechanisms by which different external factors alter episodic GnRH secretion. Note that neural inputs are conceptual and do not represent monosynaptic pathways. See the text for more details. Dep: steroid-dependent mechanisms; InD: steroid-independent mechanisms; Kiss: kisspeptin; NE: norepinephrine; Olf: olfactory epithelium; Un: unknown neurotransmitter.

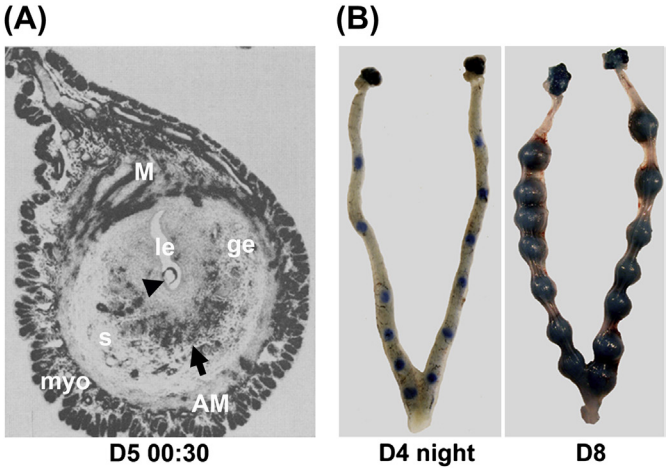

FIGURE 38.1 Increased endometrial vascular permeability upon attachment and implantation in the mouse. (A) Transverse section of uterus on day 5 (D5) early morning (00:30) of pregnancy showing implantation at the antimesometrial pole within a crypt (arrowhead). This section also shows weak alkaline phosphatase activity in the uterine stroma (black, arrow). Note the edema in the outer areas of the mucosa, contrasting with the closely packed cells around the uterine lumen (presumptive primary decidual zone). Le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; M, mesometrial pole; AM, antimesometrial pole. *(Source: Reprinted with permission from Ref. 2.)* (B) Increased vascular permeability at the sites of blastocyst attachment with the uterine luminal epithelium was detected after an intravenous injection of a blue dye (Chicago Blue B6) solution in mice at midnight of day 4 (D4) and day 8 (D8) of pregnancy. On day 4, distinct blue bands (dark bands in this picture) indicate that the attachment process has been initiated.

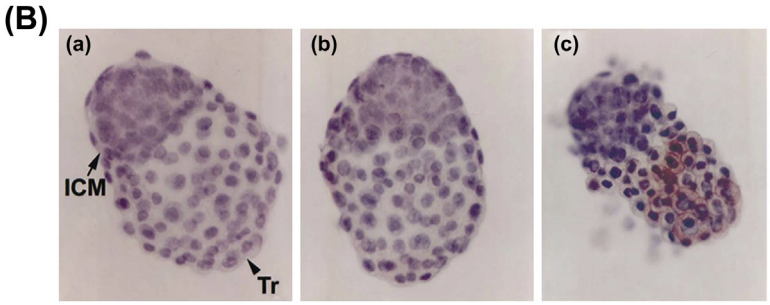

FIGURE 38.4 Molecular markers for blastocyst dormancy and activation. (B) Dormant blastocysts recovered from P_4-primed delayed-implanting mice on day 7 were cultured for 24 h in Whitten's medium, in the presence of (a) vehicle; (b) E_2; or (c) 4-OH-E_2. Blastocysts were processed for immunostaining and counterstained with hematoxylin. Red deposits indicate the sites of immunoreactive COX2. ICM, inner cell mass; Tr, trophectoderm. *Source: Reprinted with permission from Refs 67,137.*

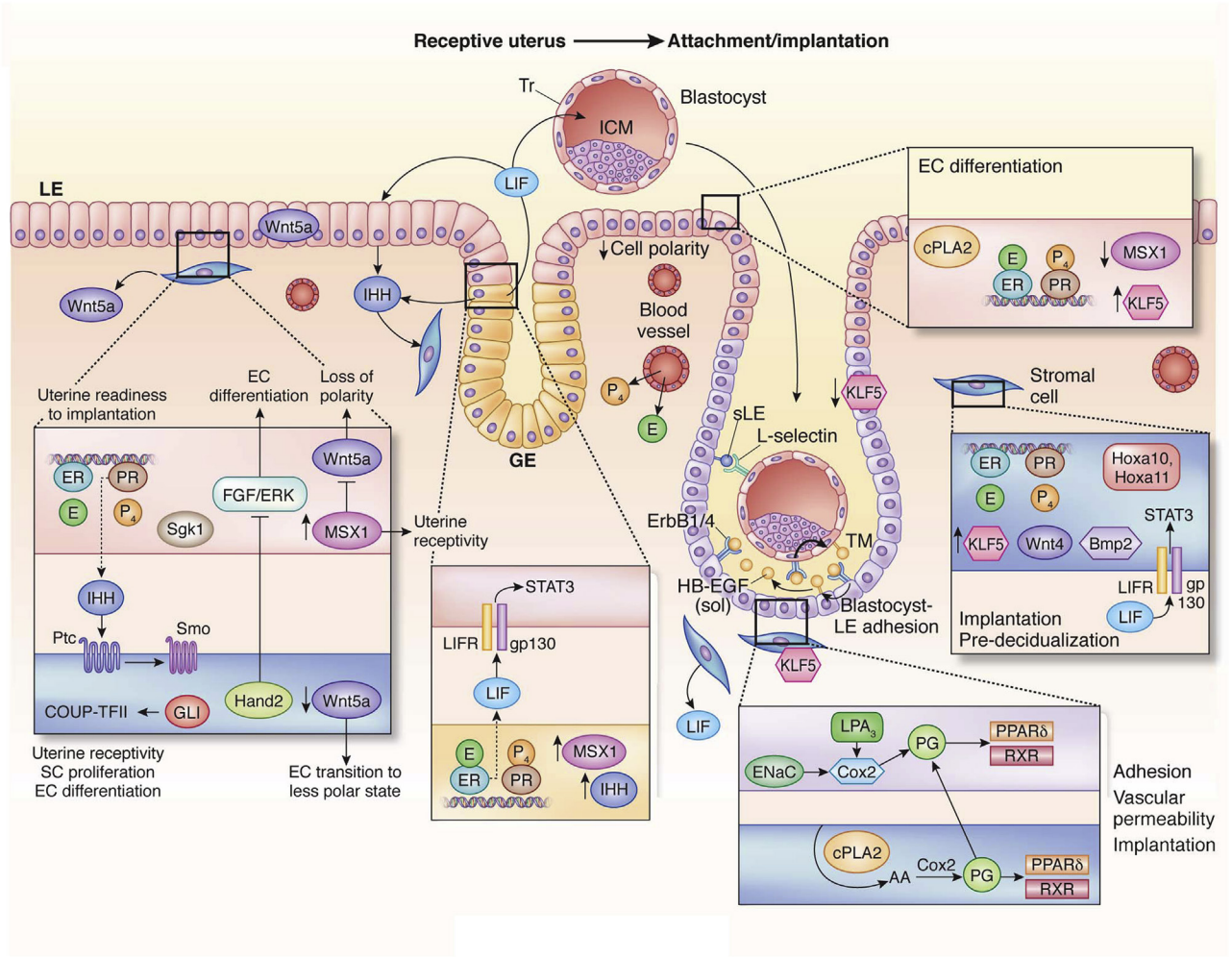

FIGURE 38.5 Signaling network for uterine receptivity and implantation. This is a hybrid cartoon, based on mouse and human studies, portraying compartment- and cell type–specific expression of molecules and their potential functions necessary for uterine receptivity, implantation, and decidualization. Interplay of ovarian P_4- and/or E_2-dependent and P_4- and/or E_2-independent factors in the pregnant uterus in specific compartments contributes to the success of implantation in a juxtacrine–paracrine–autocrine manner. During attachment, interactions between the blastocyst and luminal epithelium (LE) involve ErbB1/4 (blue) and both transmembrane (TM) and soluble (Sol) forms of HB-EGF, as well as L-selectin ligands (sLE) expressed by the luminal epithelium to L-selectin receptors on the blastocyst. The other key signaling pathways for uterine receptivity and implantation are also shown. AA, arachidonic acid; BMP2, bone morphogenetic protein 2; cPLA2α, cytosolic phospholipase A2α; COUP-TFII, chicken ovalbumin upstream promoter transcription factor 2; Cox2, cyclooxygenase 2; E, estrogens; EC, epithelial cell (luminal and glandular epithelia); ENaC, epithelium sodium channel; ER, estrogen receptor; ErbB1/4; epidermal growth factor receptor 1/4; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; GE, glandular epithelium; gp130, glycoprotein 130; Hand2, heart- and neural crest derivatives–expressed protein 2; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Hoxa10/11, homeobox A10/11; ICM, inner cell mass; IHH, Indian hedgehog; KLF5, Kruppel-like factor 5; LIF, leukemia inhibitory factor; LIFR, LIF receptor; LPA3, lysophosphatidic acid receptor 3; MSX1, muscle segment homeobox 1; P_4, progesterone; PG, prostaglandin; PPAR-δ; peroxisome proliferator-activating receptor δ; PR, progesterone receptor; Ptc, Patched; RXR, retinoid X receptor; SC, stromal cell; SGK1, serum- and glucocorticoid-inducible kinase 1; Smo, Smoothened; STAT3, signal transducer and activator of transcription 3; Tr, trophectoderm; Wnt4/5a, Wingless-type MMTV integration site family members 4/5a. Compartment colors: blue, stroma; pink, luminal epithelium; orange, glandular epithelium; purple, epithelium at the attachment site. *Source: Reprinted with permission from Ref. 153.*

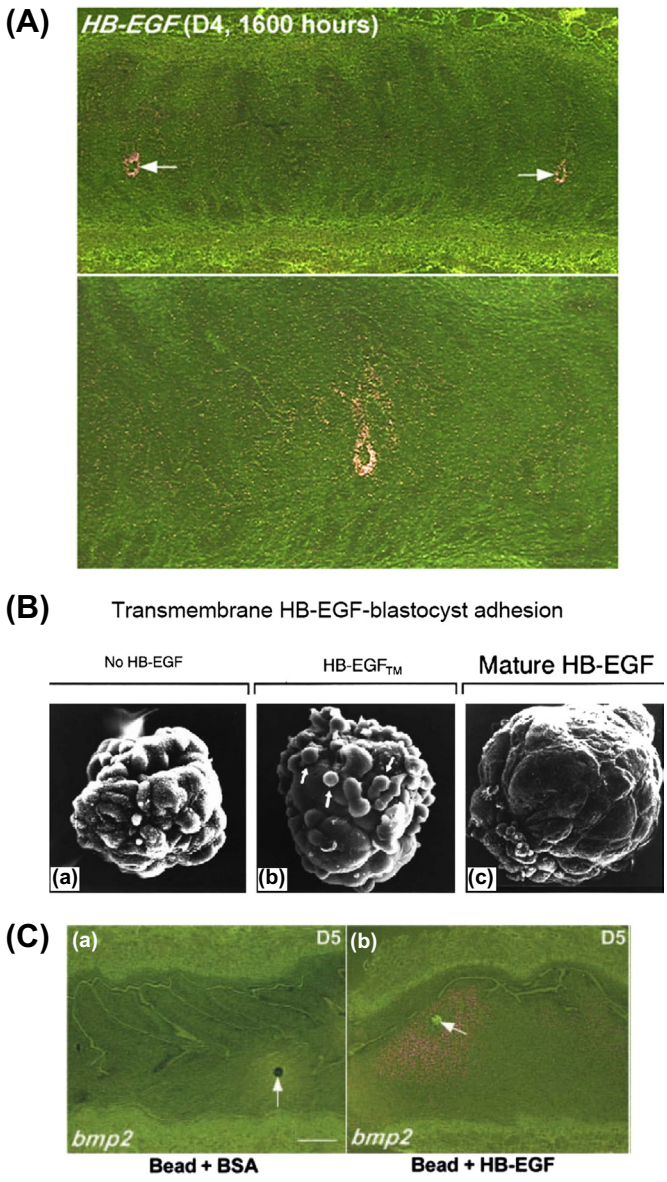

FIGURE 38.6 HB-EGF serves as a reciprocal mediator between the luminal epithelium and activated blastocyst during attachment reaction. (A) In situ hybridization of HB-EGF mRNA (dark-field) in the mouse uterus at 1600 h on day 4 of pregnancy. Note distinct hybridization signals in luminal epithelial cells surrounding two blastocysts in a longitudinal section. Arrows demarcate location of blastocysts. (B) Scanning electron microscopy of blastocysts co-cultured with 32D cells (a murine myeloid cell line). Zona-free day 4 (0900 h) mouse blastocysts were co-cultured with (a) parental 32D cells, (b) 32D cells displaying transmembrane form of HB-EGF^{TM}, or (c) 32D cells synthesizing soluble form of HB-EGF for 36 h. After extensive washing to remove loosely adhering cells, blastocysts were fixed and examined by scanning electron microscopy; magnification 800×. Arrows point to 32D cells. (C) *Bmp2* gene expression in response to beads preloaded with HB-EGF. Beads (7 beads/horn) preabsorbed either in BSA (control) or HB-EGF (100 ng/ml) were transferred into uterine lumens of day 4 pseudopregnant mice. Mice were killed on day 5 to examine *Bmp2* expression at the sites of beads. Arrows indicate the locations of the beads. Note localized stromal expression of *Bmp2* at the sites of beads preabsorbed with HB-EGF. Bar: 75 mm. *Source: Reprinted with permission from Refs 9,135,220.*

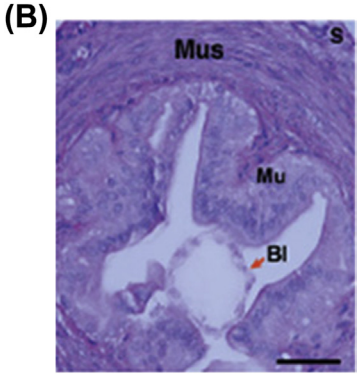

FIGURE 38.7 Impaired oviductal embryo transport causes pregnancy loss in *Cnr1*$^{-/-}$ but not *Cnr2*$^{-/-}$ mice. (B) A representative histological section of a day 7 pregnant *Cnr1*$^{-/-}$ oviduct showing an entrapped blastocyst (Bl, arrow) at the isthmus. Mus, muscularis; S, serosa; Mu, mucosa. Bar, 100 μm. *Source: Reprinted with permission from Ref. 394.*

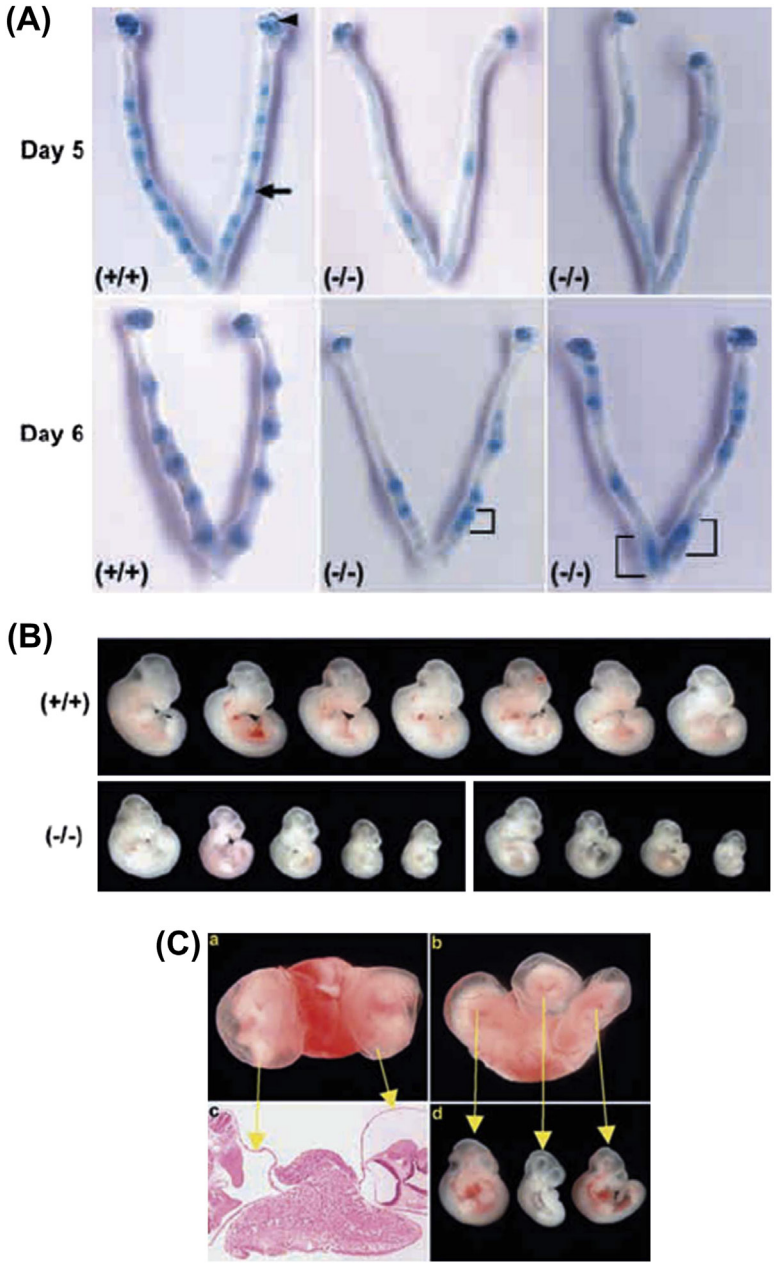

FIGURE 38.8 **Defective postimplantation development in *Pla2g4a*$^{-/-}$ mice.** (A) Representative photographs of uteri with implantation sites (blue bands) on days 5 and 6. Note very few or no implantation sites on day 5, but unevenly spaced implantation sites on day 6 in *Pla2g4a*$^{-/-}$ mice. Arrowhead and arrow indicate ovary and implantation site, respectively. Brackets indicate crowding of implantation sites. (B) Photographs of embryos isolated from implantation sites of one representative wild-type and two *Pla2g4a*$^{-/-}$ mice on day 12. Note retarded and asynchronous development of embryos in *Pla2g4a*$^{-/-}$ mice. (C) Representative photographs of conjoined embryos in a placenta (a, c) and three embryos in the same decidual envelope (b, d) from *Pla2g4a*$^{-/-}$ mice on day 12. (c) A histological section of (a) with two embryos; embryos shown in (d) are from (b). Yellow arrows indicate the source of the embryos from the decidual envelope. *Source: Reprinted with permission from Ref. 47.*

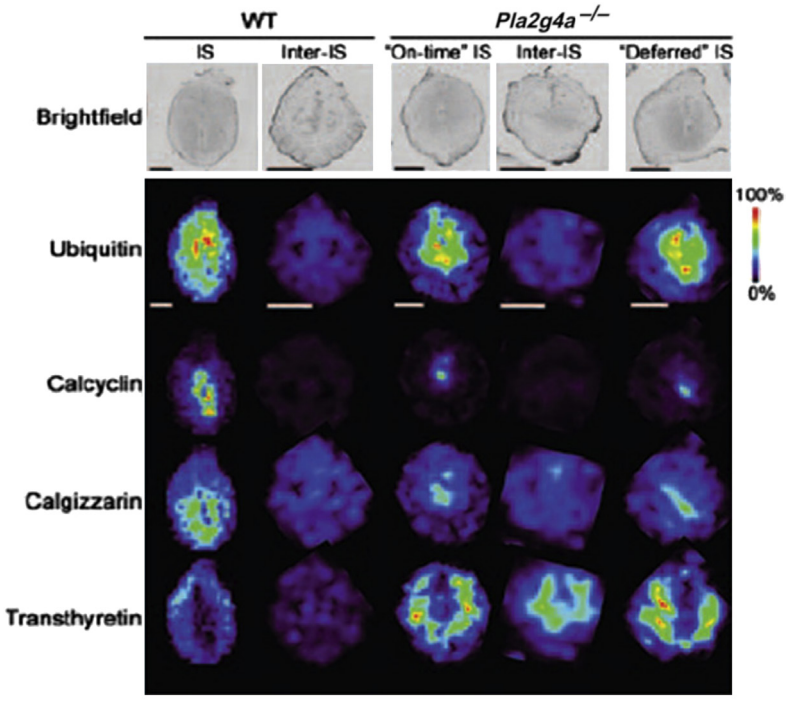

FIGURE 38.9 **Proteome profiles differ between WT and *Pla2g4a*$^{-/-}$ uteri on day 6 of pregnancy, regardless of implantation timing.** Optical images of a WT implantation site (IS) and interimplantation site (inter-IS) and *Pla2g4a*$^{-/-}$ deferred and on-time IS (*upper panel*). Bar, 670 μm. Ion intensity maps are shown below their respective bright-field images. *Source: Reprinted with permission from Ref. 480.*

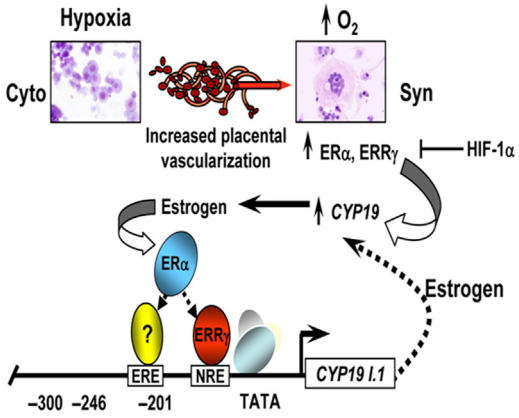

FIGURE 40.12 Model of hCYP19 regulation in human trophoblasts. Early in gestation, the placenta is relatively hypoxic; the elevated levels of HIF-1α inhibit ERRγ transcription via interaction with a response element (HRE) in the ERRγ promoter. This, together with hypoxia-mediated induction of basic helix–loop–helix factors ASCL2 and USF1/2, blocks syncytiotrophoblast differentiation and hCYP19 gene expression. After the ninth week of gestation, increased placental vascularization results in increased oxygen availability to the trophoblast cells. The consequent decrease in HIF-1α levels and the expression of other inhibitory transcription factors cause upregulation of ERRγ and induction of hCYP191.1 transcription. The increased estrogens produced, in turn, activate ERα, which may functionally interact with ERRγ and other activating transcription factors to further upregulate hCYP19 expression. *Source: Reprinted with permission from Ref. 360.*

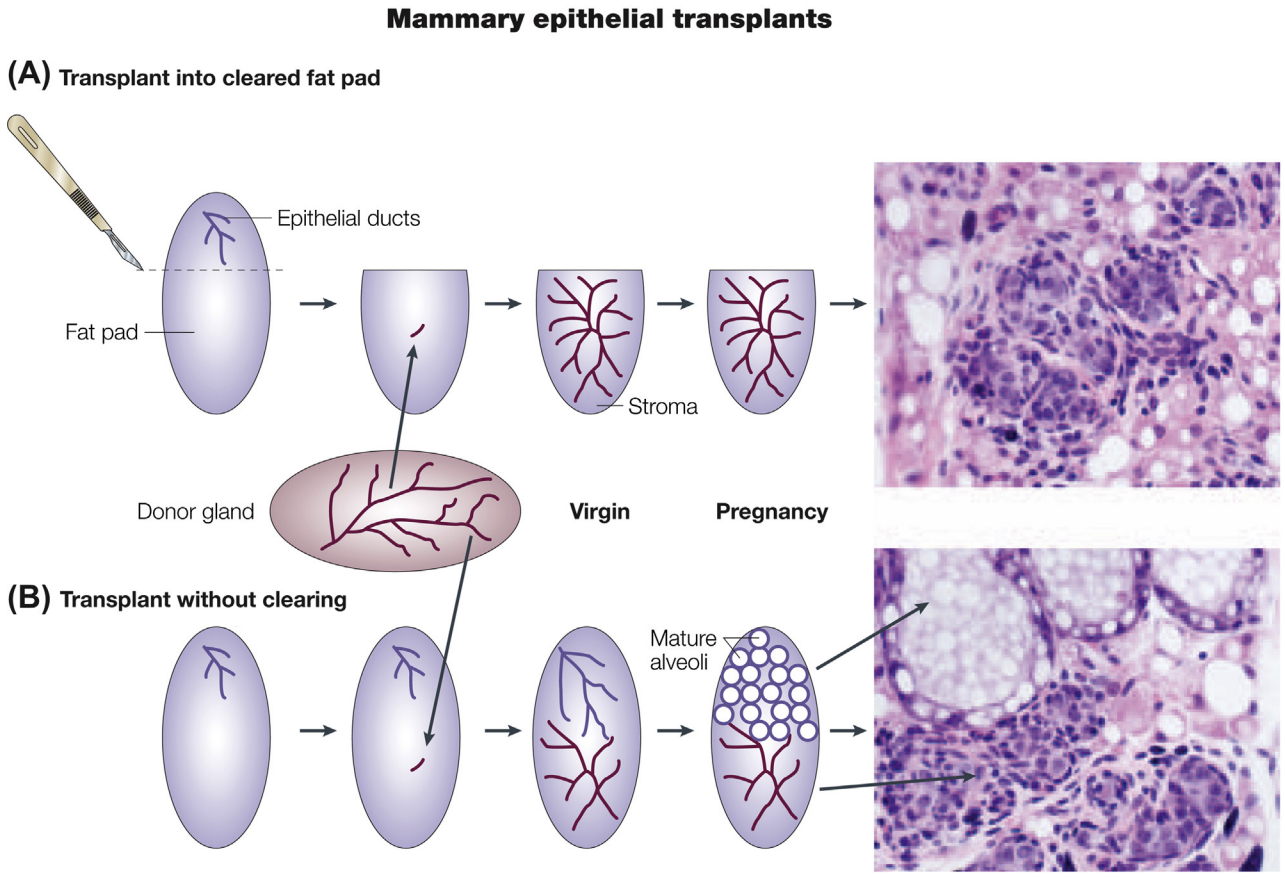

FIGURE 46.2 Use of mammary gland transplantation to assess developmental potential. (A) The area containing the ductal anlage (purple) in a 3-week-old mouse is surgically removed to generate a "cleared fat pad" into which donor tissue (dark pink) is transplanted. The development of the transplanted tissue is monitored at intervals posttransplant by either whole mount or standard histological analysis. (B) An alternative approach involves direct transplantation into a non-cleared mammary gland. Transplanted cells are frequently marked with either immune-fluorescent markers, genetic markers, or infected with viruses expressing detectable markers. *Source: Used by permission from Macmillan Publishing Company; Ref. 14.*

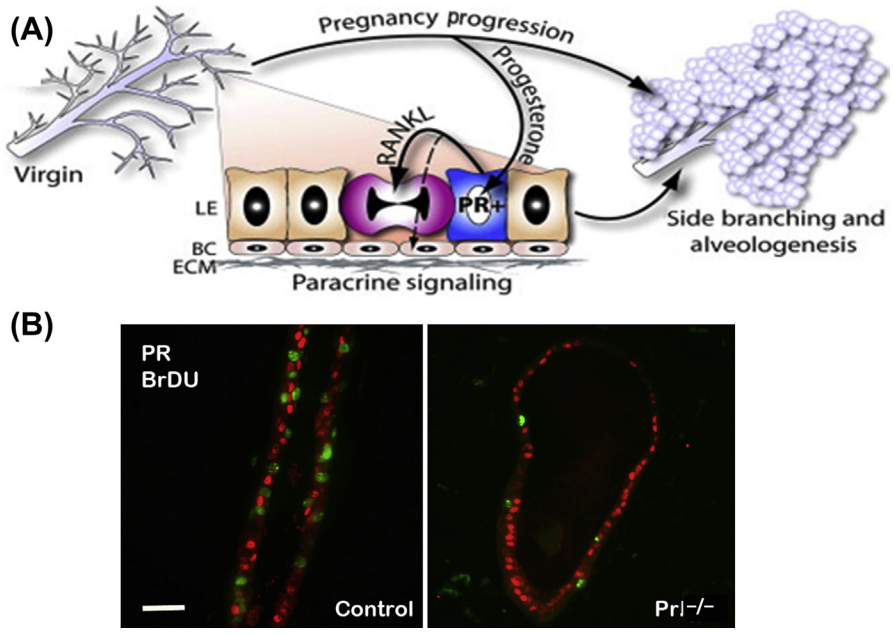

FIGURE 46.4 Role of paracrine signaling in alveologenesis. (A) As pregnancy progresses side branches develop at discreet intervals along the ducts; cells in these side branches proliferate to form alveoli. The progesterone receptor (PR) is expressed in a subset of alveolar cells that are stimulated by increasing P4 from the ovaries to secrete RANKL. This paracrine factor acts on neighboring cells to promote proliferation. (B) Expression of PR and proliferating cells after 2 days of treatment with E2 and P4 in wild-type (control) and PRL-null mice. Proliferating cells (BrDU labeled, green) and PR positive cells (red) are generally not coincident. Note that the number of proliferating cells is reduced in the absence of PRL. Scale bar, 50 μm. *Source: (A) Used by permission from Ref. 93. (B) Original figure from Ref. 64.*

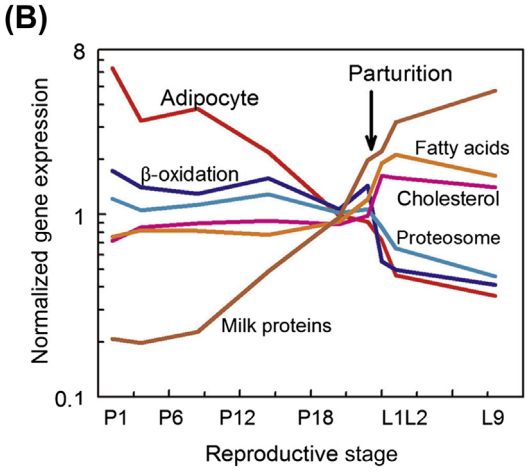

FIGURE 46.5 Changes in the differentiated activity of the mammary gland in pregnancy and lactation. (B) Changes in gene expression of different categories of genes in the mouse mammary gland from microarray studies. Expression of adipocyte-specific genes and collagens (not shown) decreases six- to eightfold during pregnancy and another twofold at parturition, whereas the genes for fatty acid degradation and many components of the proteosome are level during pregnancy and decrease about twofold at parturition. Milk protein genes, on average, increase about fivefold during pregnancy and another threefold around parturition, whereas the genes for fatty acid and cholesterol synthetic enzymes increase about twofold just after parturition. Normalized data for each class were averaged to produce the lines on this graph. *Source: (B) From Ref. 11.*

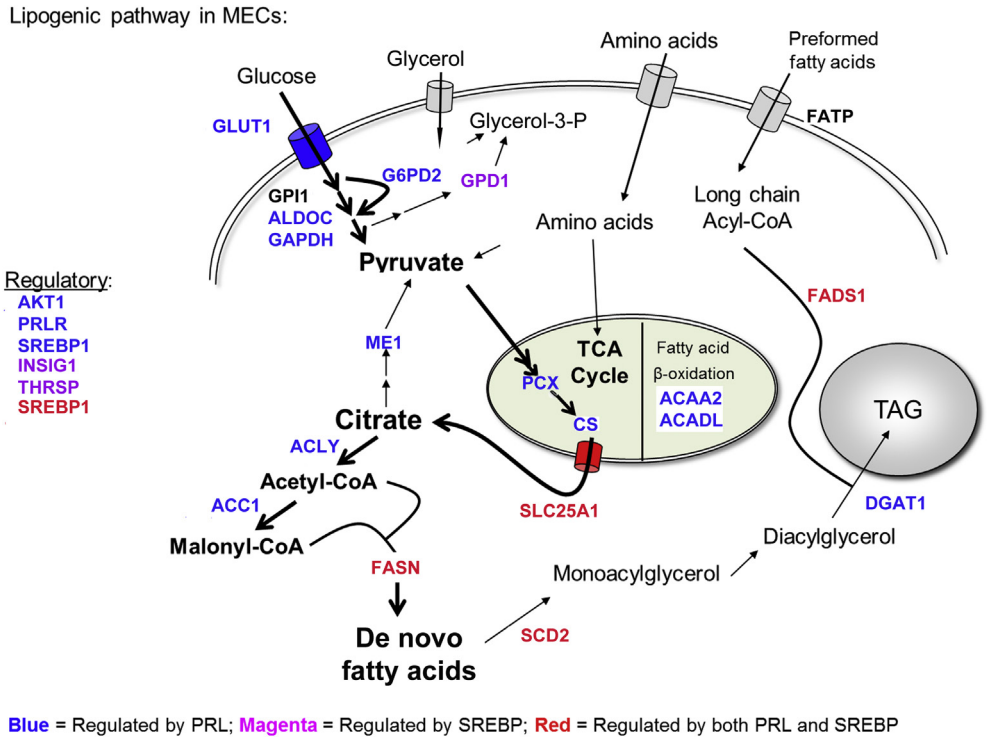

FIGURE 46.7 **Lipid synthesis pathways in mammary epithelial cells during lactation.** Substrates for lipid synthesis enter the cells via the glucose transporter (GLUT1), a glycerol transporter, as amino acids, or as preformed fatty acids via a fatty acid transport protein (FATP). Glycolysis leads to the production of both glycerol-3-phosphate and pyruvate from glucose. The genes for several enzymes in this pathway, fructose bis-phosphate aldolase (ALDOC) and glycerol-3-phosphate dehydrogenase (GAPDH), as well as glycose-6-phosphate dehydrogenase (G6PD2 in the mammary gland) are all upregulated by prolactin along with mitochondrial genes for pyruvate carboxylase (PCX) and citrate synthase (CS). Glycerol-3-phosphate is formed from dihydroxyacetone phosphate, a product of glycolysis, by glycerol-3-phosphate dehydrogenase (GPD1) to be used as a backbone for triacylglyceride (TAG) synthesis. GPD1 is regulated by SREBP. Amino acids are transformed into pyruvate and other substrates that enter the mitochondria to be transformed into citrate, which is exported via the tricarboxylic acid transporter, SLC25A1. Citrate is the major substrate for de novo synthesis of fatty acids in species other than ruminants, which utilize acetate for this purpose. Citrate is transformed into acetyl-CoA by ATP citrate lyase (ACLY), then to malonyl CoA by acetyl-CoA carboxylase (ACC1), and finally to saturated fatty acids with 8–16 carbons (C:8–C:16) by fatty acid synthase (FASN). Cytosolic malic enzyme (ME1) and the enzymes of the pentose phosphate shunt both provide the necessary reducing molecule NADPH that is required for activity of FASN. C:16 fatty acids can be desaturated by stearoyl-CoA desaturase (SCD2) prior to being esterified into monoacylglycerol, then diacylglycerol, and finally into triacylglycerols, with subsequent integration of the fatty acids derived from preformed sources. The final step in the TAG synthesis pathway is catalyzed by diglyceride acyltransferase (DGAT1). The TAG coalesce into lipid droplets. Both prolactin and SREBP have been shown to regulate the genes for FASN, SLC25A1, SCD2, and FADS1. *Source: Diagram derived from data in Refs 19,20.*

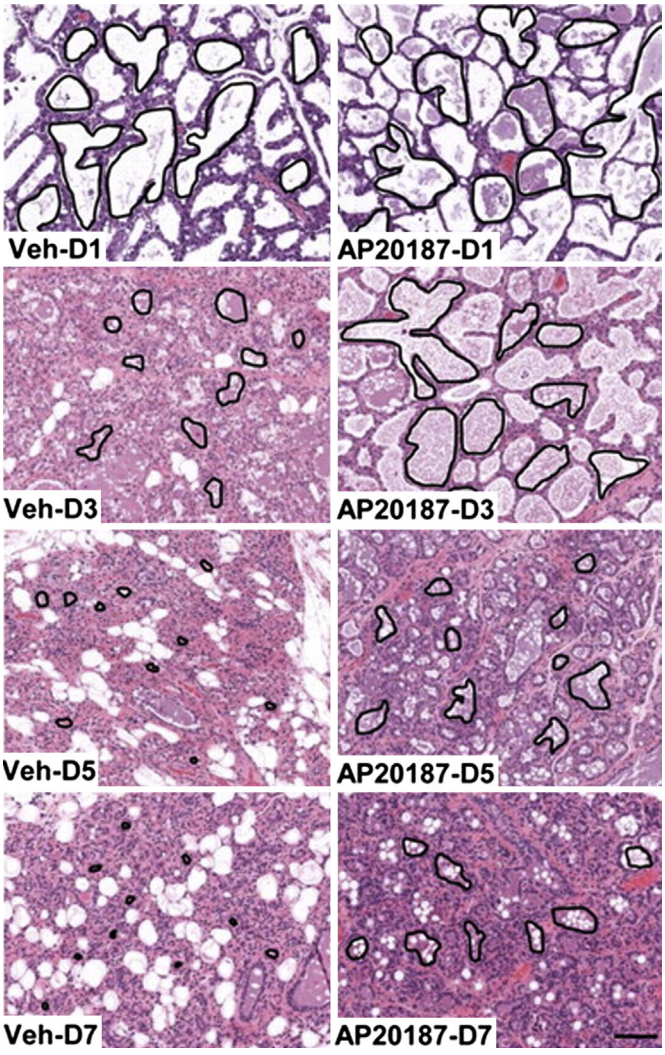

FIGURE 46.9 The role of macrophages in mouse mammary gland involution; histological analysis. Glands from Mafia mice were analyzed on days 1, 3, 5, and 7 after pup removal from a 10-day lactating mouse suckling five to six pups. Three days prior to pup withdrawal experimental dams were given a dose of AP20187, which depletes macrophages in this strain. Left-hand images, vehicle only (macrophages present); right-hand images, AP20187 (macrophages depleted). In both control and experimental mice marked luminal expansion is evident 1 day after removal of pups (some lumens outlined in black for illustration). In vehicle-only mice a marked decrease in lumen size by day three is observed as milk is resorbed. Very small lumens remain on day 7, however significant numbers of lipid filled adipocytes are evident between the alveoli starting on day three and increasing to day 7. These changes occur much more slowly in the glands of AP20197-treated mice, providing evidence that macrophages are required for several aspects of normal involution. *Source: Reproduced by permission from Ref. 183; link at Development:* dev.biologists.org.

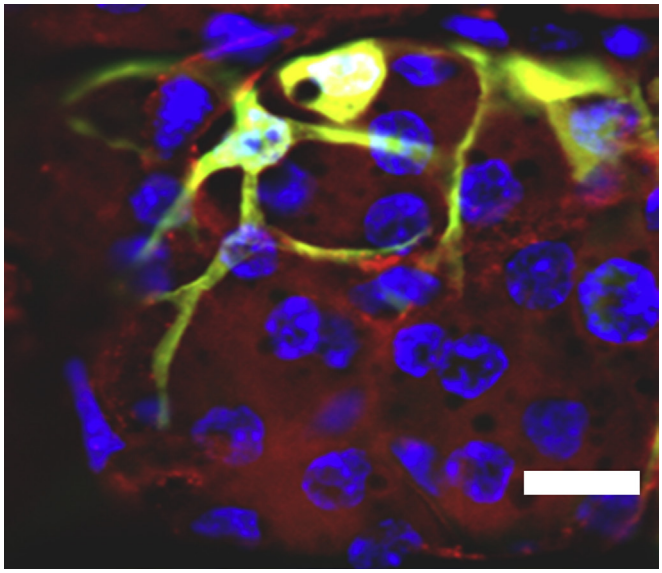

FIGURE 46.19 Myoepithelial cell in the mammary gland of a lactating mouse. The cell has been transduced with adenoviral GFP (yellow) showing processes embracing the luminal epithelial cells (red). Nuclei stained with DAPI (blue). Scale bar 10 μm. *Source: From Russell T, Fischer A, Beeman N, Freed E, Neville MC, Schaack J. Transduction of the mouse mammary epithelium with adenoviral vectors in vivo.* J Virol *2003;77(10):5801–09.* http://dx.doi.org/10.1128/JVI.77.10.5801–5809.2003, *© 2003, American Society for Microbiology.*

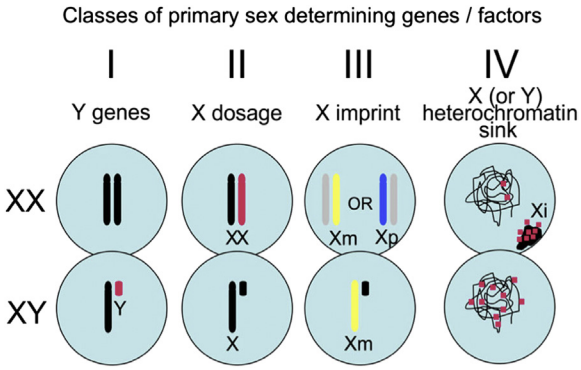

FIGURE 47.7 Four ways that sex chromosomes could affect gene expression. Four possible classes of primary sex-determining factors are recognized. Class I comprises Y genes that have a male-specific effect on one or more tissues. Class II is X genes that are expressed at a higher level in females than males by virtue of the 2:1 ratio in the number of X chromosomes. Class III is X genes that receive a parental imprint. The X chromosome receiving a maternal imprint (Xm, yellow) is active in half of XX cells and all of XY cells, whereas the X chromosome receiving a paternal imprint (Xp, blue) is active in half of XX cells and no XY cells. Class IV comprises proposed regions of the sex chromosome heterochromatin (the heterochromatic inactive X (Xi) is illustrated here) that act as sex-specific sinks for factors (red dots) that regulate the amount of euchromatin–heterochromatin at interphase and therefore affect gene expression throughout the genome. To date, specific members of only Class I have been identified (*Sry* and spermatogenesis genes). Although evidence indicates that the number of X chromosomes leads to some sex differences in phenotype, the specific genes or chromosome regions that explain these X effects have not been identified. Class IV is particularly speculative at present because it is based on a limited number of studies. Future studies are likely to expand the importance of Classes II–IV. *Source: Adapted with permission of Elsevier from Ref. 58.*

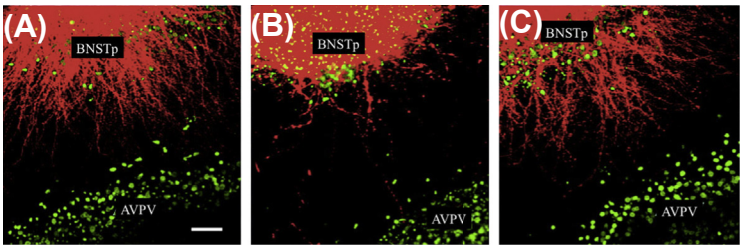

FIGURE 47.10 Target-dependent induction of neurite outgrowth in co-cultures of the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) and anteroventral periventricular nucleus (AVPV). Confocal images of BNSTp-AVPV co-cultures show DiI labeling of neuritis (pseudo-colored red) that extend from the BSTp toward the AVPV. The BSTp explant was derived from a male rat on postnatal day 5 (PN5) and co-cultured with an AVPV explant (pseudo-colored green) derived from a PN9 (A) male, (B) female, or (C) androgenized female. A marked difference in the density of neurites between the co-cultures explants was observed, with many more neuritis seen when the AVPV explant was derived from a male (A) versus a female (B) rat. Treatment of neonatal female rats with testosterone during the first 9 days of life in vivo masculinized (i.e., increased) neurite outgrowth from BSTp explants (C), suggesting that the target-dependent formation of neuritis extending from the BSTp to AVPV is determined by exposure to sex steroid hormones during the neonatal period. Scale bar: 15 μm. *Source: Adapted with the permission from Ref. 217.*

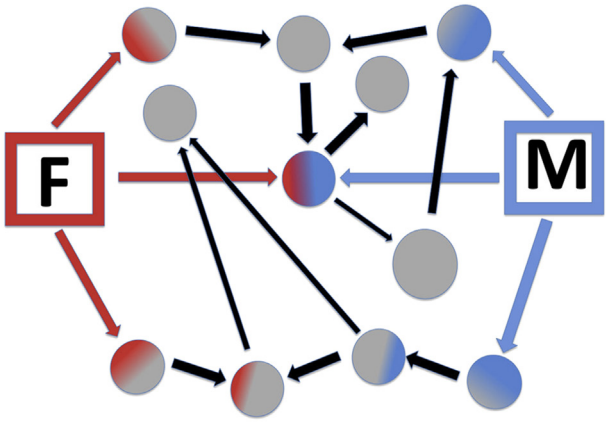

FIGURE 47.21 Schematic view of the sexome. Male- and female-biasing factors (chromosomes, hormonal profiles, etc.) are represented by the M and F boxes, respectively. These factors act on specific nodes in the gene network causing male and female bias in gene expression represented by differently colored shading. Sex bias can be propagated to other nodes, thereby increasing or decreasing gene expression and in some cases canceling out sex-biasing effects. Arnold and Lusis define the sexome as the total of all sex-biasing actions within the network.[404] Although they focus on sex bias in gene products,[404] the circles and arrows in this figure may just as well represent interactions between different organ systems. *Source: Adapted from Ref. 404.*

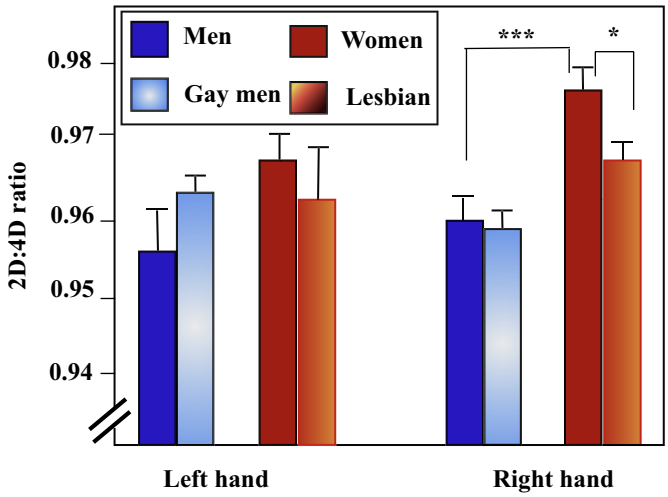

FIGURE 48.7 **Ratio of lengths of the index (2D) and ring finger (4D) as a function of sex and sexual orientation.** The 2D:4D ratio is greater in women than in men, probably due to in utero exposure to testosterone of male embryos. It is close to the male level in homosexual women, suggesting that they have been exposed to abnormally high levels of testosterone during a part of their embryonic life. For unknown reasons, these differences are more pronounced in the right hand. * = $p < 0.05$, *** = $p < 0.001$. *Source: Redrawn from Ref. 117.*

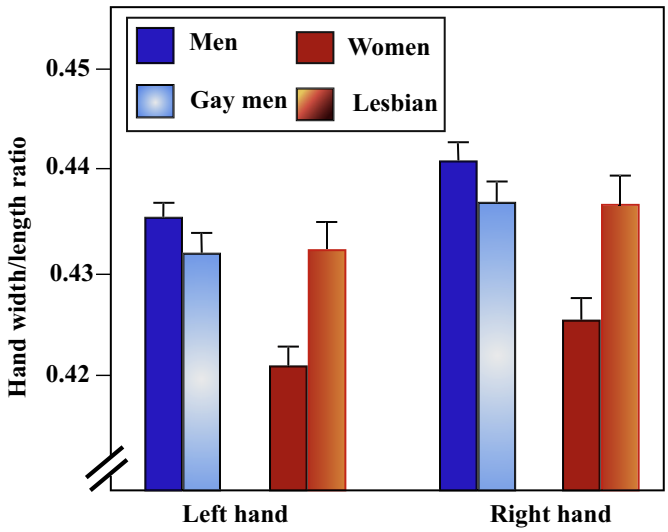

FIGURE 48.8 **Relationship between sex and sexual orientation and the ratio of width to length of the hand.** *Source: Redrawn from data in Ref. 123.*

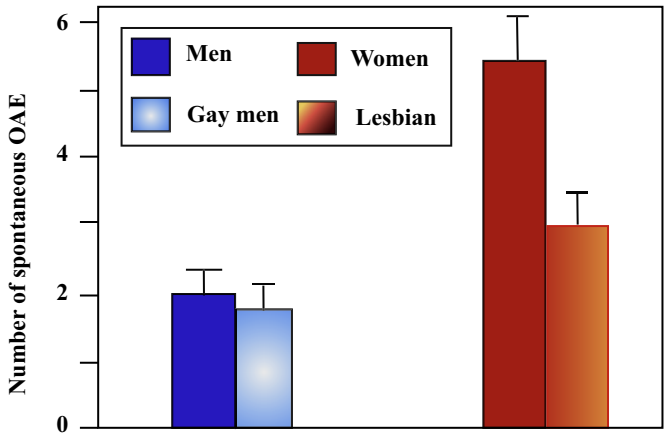

FIGURE 48.9 **Frequency of oto-acoustic emissions (OAE) evoked by click sounds measured in the right ear in men and women as a function of sexual orientation.** OAE are sexually differentiated and significantly masculinized in homosexual females. *Source: Redrawn from data in Refs 124,127.*

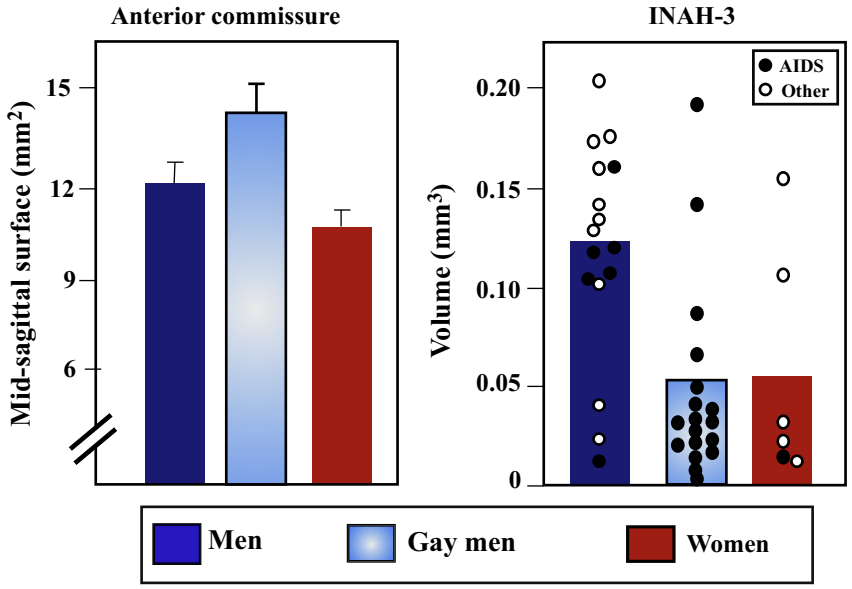

FIGURE 48.11 **Average size of the anterior commissure as estimated by its surface in the mid-sagittal plane (left) and volume of the 3rd interstitial nucleus of the anterior hypothalamus (INA-3) (right) in men, gay men, and women.** Data for the anterior commissure are presented as means ± SEM, whereas for INAH-3 the average value in each group, as well as all individual data points, are shown to clearly illustrate the partial overlap between groups despite the significant differences between men and women and between men and gay men. Individual data points are filled in subjects who died from AIDS and open in subjects who died from other causes. The smaller average size of INAH-3 in gay men cannot be accounted for by the fact that all subjects included in the study had died from AIDS since in the control group, the INAH-3 of men who died from AIDS is not smaller than in men who died from other causes. *Source: Redrawn from data in Refs 140,141.*

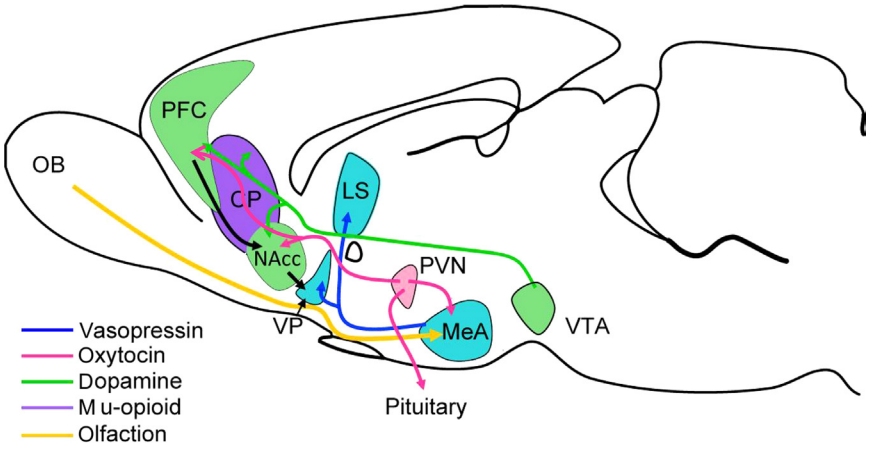

FIGURE 48.21 A schematic of the proposed neural circuitry of pair bond formation in prairie voles. Mating stimulates the release of oxytocin from neurons in the paraventricular nucleus of the hypothalamus (PVN) into the nucleus accumbens (NAcc) and prefrontal cortex (PFC) in females, and the release of vasopressin from neurons in the medial amygdala (MeA) into the ventral pallidum (VP) and lateral septum (LS) in males. Mating also stimulates the release of dopamine from neurons in the ventral tegmental area (VTA) into the NAcc and PFC. The MeA receives olfactory input from the partner and conveys the neural encoding of the olfactory signature of the partner to the striatum and LS. Mu-opioid receptors in the CP stimulate the rewarding aspects of mating. OT and AVP facilitate the processing of the olfactory signatures of the mate, and dopamine facilitates an association between the neural encoding of the social cues of the partner and the opioid-based reward and reinforcement. The actions of these neurochemicals in these circuits lead to a conditioned partner preference. *Source: Adapted from Refs 271,334.*

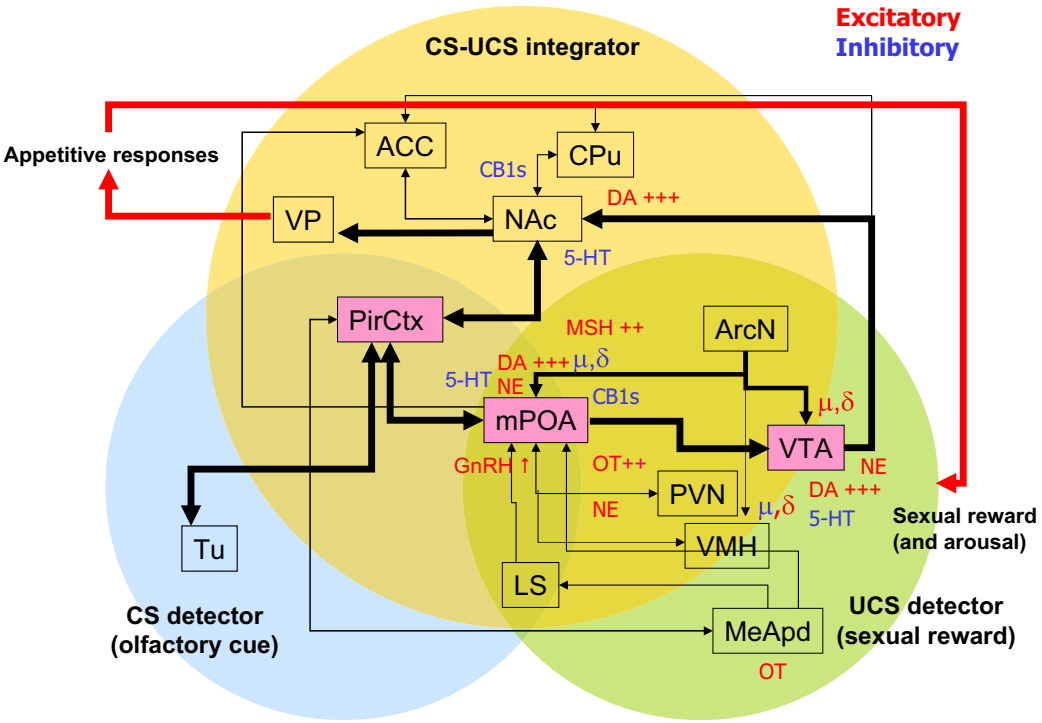

FIGURE 50.28 **Neural systems critical for the display of sexual behavior in the rat.** Appetitive behaviors made toward unconditioned or conditioned sexual incentive stimuli lead to sexual reward that is processed by three interactive systems. Two systems process olfactory stimuli and sexual reward relatively independently, whereas a third, mesolimbic DA system, acts to integrate both the conditioned olfactory cue and its rewarding sexual outcome. Three common regions, the piriform cortex, mPOA, and VTA, are activated in male and female rats by conditioned olfactory stimuli. Opioid actions in the VTA potentiate mesolimbic DA activation, whereas opioid actions in the mPOA inhibit sexual arousal and desire. Neurotransmitter systems or their receptors in red are excitatory for sexual motivation whereas those in blue are inhibitory. Black pathways signify major inputs and outputs of the system. Note that opioids can be excitatory in the VTA, inhibitory in the mPOA, or either in the VMH (depending on the receptor type). A similar system is activated in humans.[63] Abbreviations: ACC, anterior cingulate cortex; ArcN, arcuate nucleus of the hypothalamus; CB1, cannabinoid Type 1 receptor; CPu, caudate-putamen (striatum); DA, dopamine; δ, delta opioid receptors; GnRH, gonadotropin releasing hormone; LS, lateral septum; MeApd, posterior-dorsal nucleus of the medial amygdala; mPOA, medial preoptic area; MSH, melanocyte stimulating hormone; μ, mu opioid receptors; NAc, nucleus accumbens; NE, norepinephrine OT, oxytocin; PirCtx, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; Tu, olfactory tubercle; VMH, ventromedial nucleus of the hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area; 5-HT, serotonin. *Source: Adapted from Pfaus et al.*[683]

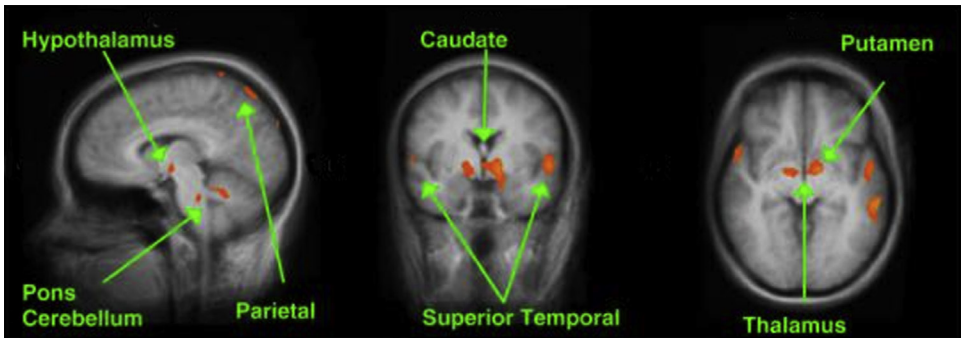

FIGURE 51.20 Sites of the maternal brain where fMRI activity was higher (indicated by red) in mothers who delivered vaginally compared to via Caesarean section while listening to their own infant crying. *Source: Modified from Swain et al., 2008.*[98]

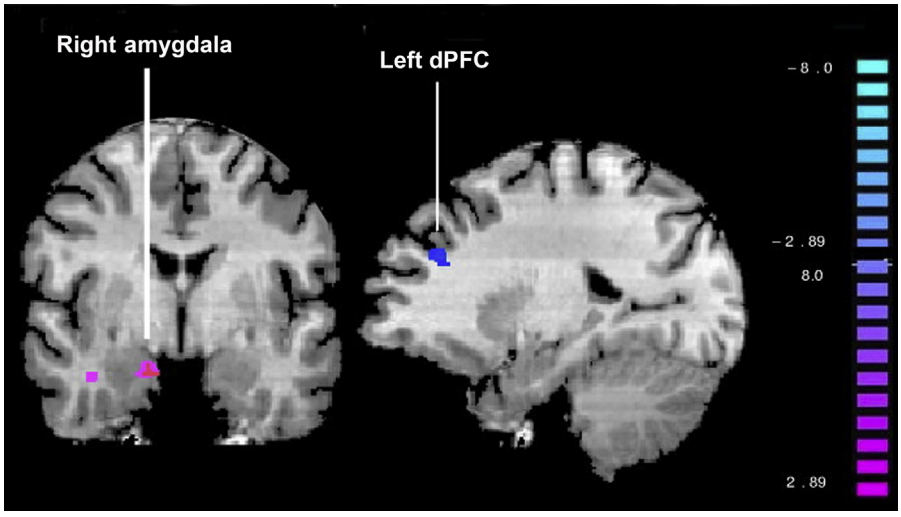

FIGURE 51.21 Sites in the parental brain where mothers (left) or fathers (right) showed greater fMRI activity in response to their own infant video compared to viewing an unfamiliar infant. Mothers showed greater activity in the right amygdala and temporal, occipital, and parietal cortices compared to fathers, while fathers had greater activity in the dorsal prefrontal cortex (dPFC). *Source: Modified from Atzil et al., 2012.*[711]

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