The Biology of Early Influences

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PREFACE

The underlying theme of this book is the role of experience in the development of the nervous system. It is now widely acknowledged that there are a variety of organism–environment interactions that guide the development of the nervous system. The popular press has also picked up on this theme and parents are being advised to enrich their child's sensory experience as early as possible. The importance of experience in development is so well accepted that it has begun to dictate public policy. In 1997, the President and First Lady hosted "The White House Conference on Early Childhood Development and Learning: What New Research on the Brain Tells Us About Our Youngest Children." Along with this conference came a reemphasis on programs directed at enriching the environment of America's youth. In some cases laws have been passed to assure enhanced early experience. In Georgia, the former governor proposed sending newborn children home from the hospital with classical music CDs. This received some praise, but was also ridiculed by others who felt the policy was not justified by the available scientific evidence.

Here in Florida, enhanced "brain development activities" are mandated for state-funded child care facilities. Chapter 402.25 of the 1998 State of Florida Statues reads (emphasis added):

> Infants and toddlers in state-funded education and care programs; brain development activities.—Each state-funded education and care program for children from birth to 5 years of age **must provide activities to foster brain development in infants** and toddlers. A program must provide an environment rich in language and music and filled with objects of various colors, shapes, textures, and sizes to stimulate visual, tactile, auditory, and linguistic senses in the children and must include classical music and at least 30 minutes of reading to the children each day.... A program must provide training for the infants' and toddlers' parents including direct dialogue and interaction between teachers and parents **demonstrating the urgency of brain development in the first year of a child's life**. Family day care centers are encouraged, but not required, to comply with this section.

Whether it is exposure to Mozart or foreign language, there is a confidence that these early experiences enhance brain development and make for a more intelligent adult. This commonly accepted folklore regarding the importance of early sensory experience does have some scientific backing. One of the classic examples comes from the early work on differences between rats raised in an "enriched" environment and those raised in

"impoverished" environments (see Renner and Rosenzweig, 1987 for review). Such studies demonstrated, for instance, that animals perform better at maze learning tasks when raised in "enriched" environments which contain complex sensory stimuli, allow for social interactions, and permit a greater variety of motor behaviors (Forgays and Forgays, 1952). Additionally, the enhanced rearing conditions lead to morphological signs of more robust brain development, such as larger brains and more dendritic branches in cortical neurons (Globus *et al.*, 1973). Although the differences between groups are commonly viewed as "enrichment" effects, they could just as easily be viewed as deprivation effects due to raising the "impoverished" rats in the sterile environment of the typical rat home cage. In fact, it can reasonably be argued that even the "enriched" condition is relatively impoverished compared to the rich natural environment in which the rat evolved. Nevertheless, whether viewed as enrichment or deprivation effects, the fact that differences between groups emerge can be taken as evidence that experience plays a key role in regulating brain and behavioral development.

A less semantic question regarding the effects of these enrichment experiments is, *how* do these differences emerge? What is it about the early experience that enhances (or prevents reduction in) brain growth? Clearly the complex constellation of differences between enriched and impoverished rearing conditions makes this question difficult to answer. A fruitful approach to get at the more mechanistic biological questions comes from studies in which the manipulation of experience is defined more concretely. This is commonly accomplished by restricting early experience in a single sensory modality. Depriving the subject of sensory experience within a specific modality provides a cleaner definition of the exact manipulation of experience than is possible when using the more general "enrichment" manipulation. This allows a focused analysis on the specific sensory system, and the biological mechanisms underlying these changes can be explored in a systematic manner. Probably the most famous of this type of experiment are the studies of Hubel and Wiesel on the effects of altering early visual experience (Wiesel and Hubel, 1963a,b; Wiesel and Hubel, 1965; Hubel and Weisel, 1970; Hubel *et al.*, 1977). The work on the visual system stemming from these landmark studies has been frequently and extensively reviewed elsewhere (Lam and Shatz, 1991; Katz and Shatz, 1996; Crair *et al.*, 1998). Consequently, in this volume we emphasize what has been learned from studies of a variety of other model systems.

For the purposes of this book, "experience" is broadly defined to include neural activity, hormonal environment, social interactions, as well as exposure to sensory stimuli. As will be discussed in each of the chapters, these early factors have dramatic effects on the ultimate fate and function of neurons. While it is no longer necessary to ask "if" experience influences brain development, much is left to be known "how" experiential influences exert their effect. Thus, a common theme in the work of all of the chapters is the biological mechanisms of early experiential influences in neural development. The word "early" (as in "early influences") is, of course, a relative term. With this in mind, the chapters in this book are organized into 3 sections loosely based on the time at which the experiential manipulation is typically performed. The first section contains chapters that examine the lifelong influences of embryonic or perinatal manipulations, exemplified by studies on the motor, auditory and gustatory systems. The second section deals with the changes that are produced by manipulations early after birth or hatching and these chapters focus on the olfactory and auditory systems. The final section examines changes during later developmental periods and, in particular, during major periods of transformation in the organism's life. Here, the model systems include moths, frogs, birds, and crayfish.

One purpose of our symposium was to bring together the ideas of investigators who use a variety of model systems to study the biology of early influences. Consequently, in this compilation we have intentionally juxtaposed work using different model systems, but which share common themes related to the development of the nervous system. It is hoped that the breadth of approaches will lead the reader to a greater depth of understanding and broader appreciation for the possible mechanisms by which environmental factors influence neural development.

Discussion of the importance of early experience is prevalent in the current public and scientific forums. While media, public health advocates, parents, and even legislators are all trumpeting the need for enriching our children's lives, it is not the primary goal of this volume to inform public policy. However, given the recent extensive air play on the importance of early experience in brain development, we felt it was time for us to evaluate critically what we know about the biological underpinnings of early influences.

Richard Hyson
Frank Johnson

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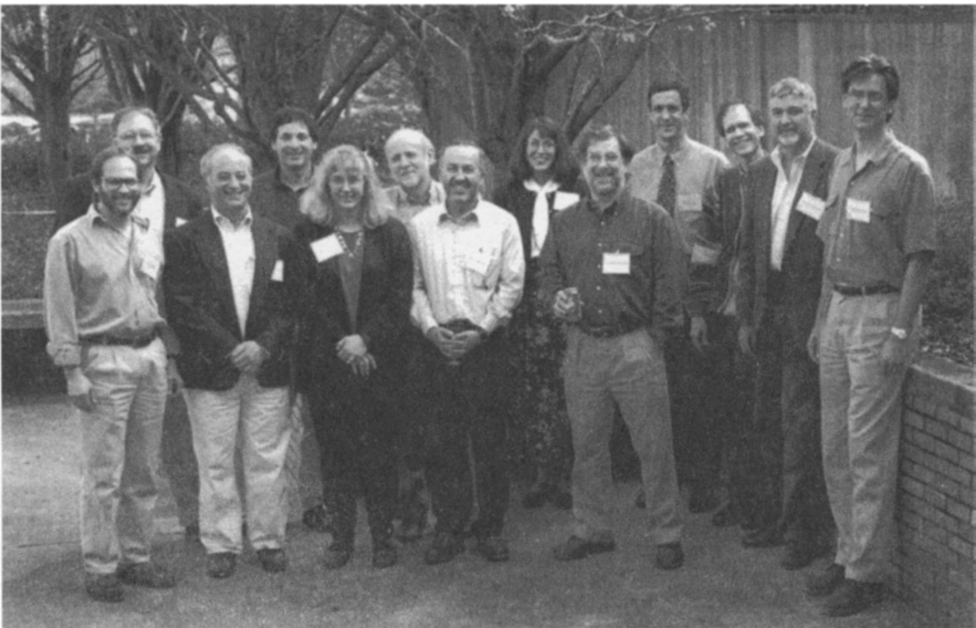
The chapters in this volume were submitted by participants in a symposium entitled "The Biology of Early Influences" that was held in Tallahassee in March of 1998. The main goal of this symposium was to better understand the biological mechanisms by which early experience influences neural development. Towards this end, we brought together investigators who study a variety of model systems for a weekend of instructive and lively discussion. This symposium was part of the annual Rushton lecture series sponsored by the Program in Neuroscience at the Florida State University thanks to funding from the College of Arts and Sciences. Partial support for this symposium was also provided by the Congress of Graduate Students at the Florida State University. The annual lecture series is named for eminent neuroscientist W. A. H. Rushton, who was formerly a regular visitor to FSU. Additional information about the Program in



Participants at the 1998 Rushton Symposium: The Biology of Early Influences. Front row (left to right): Dan Sanes, Edwin Rubel, Janis Weeks, Robert Contreras, Donald Edwards. Back row: Michael Stryker, Michael Leon, Ronald Oppenheim, Gail Burd, David Hill, Richard Hyson, Thomas Parks, Frank Johnson.

Neuroscience and Rushton lecture series can be obtained via the Internet at http://www.neuro.fsu.edu/. We as organizers and editors would like to thank the Program for Neuroscience, comprised of the departments of Psychology and Biology of the College of Arts and Sciences, and the department of Nutrition, Food and Exercise Sciences of the College of Human Sciences. We also thank Kathleen Carr for editorial assistance and MaryAnn McCarra at Kluwer Academic/Plenum Publishers for her assistance (and patience) in publishing these proceedings.

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1

MECHANISMS OF ACTIVITY-DEPENDENT MOTONEURON DEVELOPMENT AND SURVIVAL IN THE CHICK EMBRYO

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1. ONSET OF NEURAL FUNCTION AND BEHAVIOR

Neurons in the central and peripheral neurons system become capable of generating axon potentials, neurotransmitter release and synaptic transmission prior to their complete differentiation and in some cases this functional activity begins at remarkably early stages of embryogenesis (Provine, 1973; O'Donovan, 1999; Milner and Landmesser, 1999). Overtly this neuronal function is manifested as embryonic and fetal movements and reflexes that have been the focus of considerable research over the past century (Hamburger, 1963; Oppenheim, 1982; Gotttlieb, 1973; Michel and Moore, 1995). The developmentally early appearance of neuronal activity and behavior raises the obvious question of what adaptive role, if any, is served by prenatal neurobehavioral function. Early neural activity may be an epiphenomenon, in that it merely indicates that neuronal differentiation is proceeding normally. Alternatively, this early function may be a necessary feature of early nervous system organization acting to prepare the nervous system for its later role in mediating complex behavioral patterns (Crair, 1999). Finally, early neurobehavioral function may serve some immediate developmental function, a role I have previously called ontogenetic adaptations (Oppenheim, 1981; Hall and Oppenheim, 1986). For example, neuromuscular function and embryonic movements are known to play a role in the normal differentiation of skeletal muscles, synovial joints, lung differentiation and synapse formation between motorneurons and muscle. In the course of attempting to examine what role neuromuscular activity plays in the chick embryo, we discovered another apparent ontogenetic adaptation-like role for early neural function. Chronic activity blockade of chick embryos during a critical period between embryonic day (E) 5–6 and E10 resulted in a dramatic increase in the number of spinal motoneurons (MNs) (Fig. 1) and the hyperinnervation of skeletal muscle (Pittman and Oppenheim, 1978, 1979; Oppenheim and Chu-Wang, 1982).

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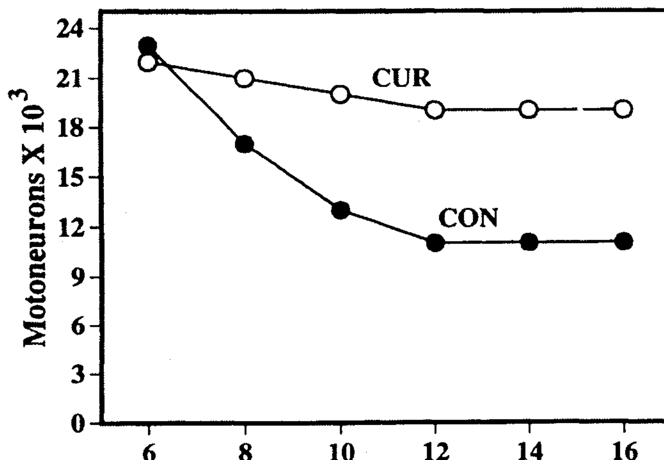

Figure 1. The number of surviving lumbar motoneurons following daily treatment from E5 to E12 with paralytic doses of curare (see Pittman and Oppenheim, 1979). Open symbols = curare; closed symbols = saline control.

2. NEURONAL CELL DEATH

The overproduction of cells and their subsequent reduction by programmed cell death (PCD) is a common theme during the development of most tissues and organs, including the nervous system (Glucksmann, 1951; Oppenheim, 1998; Raff, 1992). Approximately one-half of all initially generated spinal and cranial MNs (as well as most other central and peripheral neurons) undergo a period of PCD during the time when they are forming provisional synaptic interactions with their target cells (Hamburger, 1975; Oppenheim, 1998). Historically, the most popular explanation for neuronal survival and death in this situation has been the neurotrophic hypothesis, whereby neurons are thought to compete for uptake (at nerve endings) of limiting amounts of target-derived survival-promoting molecules (Hamburger and Oppenheim, 1982; Purves, 1988). The losers in this competition undergo PCD and the winners survive and complete their differentiation. Over the past ten years it has become clear that the death of neurons in this situation is an active process requiring the expression of cell death genes and their protein products that then induce degeneration, and that cell survival, by contrast, involves active suppression of this death pathway by distinct survival-promoting genes (Oppenheim, 1999).

It follows from the assumptions of the neurotrophic hypothesis that it is the failure of some developing neurons (i.e. those that normally die by PCD) to obtain sufficient trophic factor that triggers the intracellular events that then induces their degeneration. Indeed for some neuronal populations (e.g., sensory neurons) this prediction has been rigorously tested and shown to be true. For example, by providing excess amounts of exogenous or endogenous sources of neurotrophins it is possible to rescue most or all sensory neurons from normal PCD (Snider, 1994; Caldero *et al.*, 1998). The conclusion from these studies is that the synthesis or production of trophic factors by targets is, indeed, normally limiting and that by experimentally increasing these amounts to near saturating levels sensory neurons destined to die are rescued.

In contrast to the situation for sensory neurons, however, the application of the

classic neurotrophic hypothesis to MNs is not so clear-cut (Oppenheim, 1989). This is partly owing to the fact that the target-derived trophic molecules for MNs are not as well characterized as for sensory neurons, and partly to the fact that neurons, like MNs, that have their cell bodies in the central nervous system (CNS) have the potential for more complex interactions with non-target-derived trophic factors for promoting MN survival (Oppenheim, 1996).

The activity blockade experiment described above, in which neuromuscular blockade rescues MNs from PCD, offers one means for testing the applicability of the neurotrophic hypothesis to MNs. The simplest explanation for the rescue of MNs in that study is that synaptic or muscle inactivity results in an up-regulation of muscle-derived trophic factors for MN survival (Fig. 2; the *production* hypothesis, see Oppenheim, 1989).

If correct, then the prediction from the *production* hypothesis is that muscle taken from activity blocked embryos should contain more MN survival-promoting trophic activity than normally active, control muscle. However, two independent studies testing this hypothesis have given negative results. Using soluble fractions of muscle extracts derived from active *vs* inactive embryos it was found that MN survival *in vitro* and *in vivo* was indistinguishable (Tanaka, 1987; Houenou *et al.*, 1991). That is, although muscle extract promoted MN survival *in vivo* and *in vitro*, thereby supporting the idea that muscle is an important source of trophic factors for MN survival, the "inactive" extract was no more effective than "active" extract. Although these data are not consistent with the classic neurotrophic ("production") hypothesis, one weakness of these experiments is the failure to actually measure protein levels of known muscle-derived MN trophic molecules in the active *vs* inactive muscles. However, as noted above, until such factors have been identified and better characterized, meaningful experiment are not yet possible.

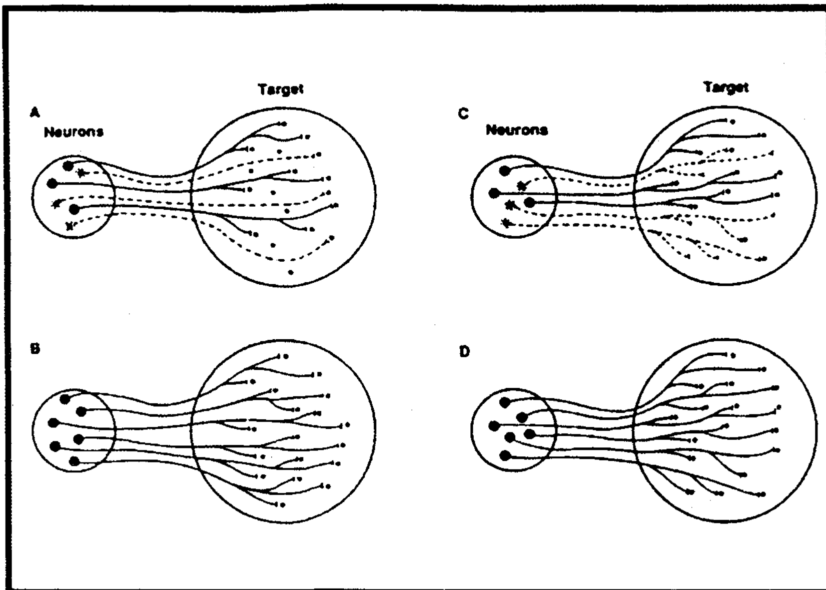

Figure 2. Schematic illustration of the access (A,B) and production (C,D) hypotheses in control (A,C) and activity-blocked embryos (B,D). MNs complete via axonal branches and synaptic terminals in the target for neurotrophic molecules (black dots). Activity blockade either increases trophic factor *production* or increases MN *access* via branching and synapse formation.

3. ACTIVITY BLOCKADE AND NEUROMUSCULAR INNERVATION

Assuming for the time being then, that muscle inactivity does not increase the production of target-derived trophic factors, what alternative hypothesis might help to explain how inactivity rescues virtually all MNs from PCD? The initial observation that activity blockade regimens that promote MN survival also result in muscle hyperinnervation (Pittman and Oppenheim, 1979; Oppenheim and Chu-Wang, 1983) provides one explanation. Namely, that activity blockade somehow induces MNs to form more synaptic connections with muscle, thereby allowing all or most MNs, including the 50% that normally undergo PCD, to gain access via nerve terminals to muscle-derived trophic support that is normally unavailable to them (Fig. 2; the *access* hypothesis, Oppenheim, 1989). The *access* hypothesis provides a means for explaining increased MN survival without assuming an activity-dependent regulation of trophic factor synthesis or production. In fact, one could argue according to this scheme that normally there is sufficient trophic factor production for *all* MNs but that access is the critical limiting event in the competitive process.

One weakness of this explanation, based on our original observations (Pittman and Oppenheim, 1979; Oppenheim and Chu-Wang, 1983), is that owing to technical limitations at the time, it was only possible to assess hyperinnervation a few days *after* the cessation of normal cell death (i.e. on E14–E16) in both normal and activity blocked embryos. For this reason it was not possible to ascertain whether hyperinnervation was a cause or a result of increased MN survival.

However, this problem was addressed in a later series of elegant studies by Lynn Landmesser and her colleagues. They were able to demonstrate that activity blockade dramatically increased MN axonal branching and synapse formation when embryos were examined early in the MN cell death period (i.e. on E6–E7) before any appreciable MN loss occurs (Dahm and Landmesser, 1988, 1991; Landmesser, 1992). From these data one can conclude that intramuscular branching, synapse formation and hyperinnervation are early events following activity blockade that precede the rescue of MNs and, therefore, are more likely to be the cause rather that the effect of increased MN survival. Further support for this possibility comes from a more recent experiment by Landmesser and her colleagues. They showed that by perturbing (reducing) axonal branching and synaptogenesis by a means independent of neuronal activity, using reagents that alter the function of adhesion molecules such as N-CAM and L1, that MN survival was significantly reduced (Tang and Landmesser, 1993). Collectively then, these various lines of evidence suggest that perturbations of intramuscular nerve branching and synaptogenesis per se, whether achieved by activity blockade or by other means, may alter the access of MNs to muscle-derived survival factors.

4. ACTIVITY-DEPENDENT MUSCLE-DERIVED NERVE BRANCHING FACTORS

The observation that one of the early events induced by activity blockade in the chick embryo is increased nerve branching and nerve-muscle contacts, suggests that one of the roles of embryonic neuromuscular activity is the regulation of a muscle-derived nerve sprouting or branching factor that only indirectly promotes MN survival, as proposed in the “access” hypothesis. Although there are also other alternative explanations

for these events, the simplest explanation is to assume the existence of an activity-dependent, muscle-derived MN-specific sprouting molecule.

One promising candidate for such a molecule is the insulin-like growth factors, IGF-1 and IGF-2 (IGFs). IGFs are expressed in muscle and have been implicated in the activity-dependent sprouting and regeneration of postnatal and adult rat MNs (Ishii, 1989; Caroni and Grandes, 1990; Caroni and Becker, 1992; Caroni and Schneider, 1994; Caroni *et al.*, 1994). IGF receptors are expressed on chick embryo MNs, IGFs can promote neurite growth and branching of cultured chick MNs (Caroni and Grandes, 1990; Caroni *et al.*, 1994) and IGFs promote chick MN survival *in vivo* (Neff *et al.*, 1993). To further explore the role of IGFs in regulating MN survival in the chick embryo, we have carried out a series of *in vitro* and *in vivo* studies designed to test some of the major predictions suggested by the IGF hypothesis (D'Costa *et al.*, 1998).

The first question we addressed is whether IGFs act as sprouting or survival factors (or both) in the chick neuromuscular system. If, in fact, IGFs are survival factors, then they should promote the survival of MNs *in vitro*, even in the absence of skeletal muscle targets. However, we have failed to observe any consistent survival effects of IGFs on cultured chick MNs (Fig. 3) (D'Costa *et al.*, 1998), even though under similar conditions *in vitro*, IGFs do promote neurite growth and branching (Caroni *et al.*, 1994). From these data, we conclude that the *in vivo* survival promoting effects of IGFs on MNs (Neff *et al.*, 1993) are indirect and may instead be mediated by IGF actions on nerve growth and branching.

To test the role of IGFs in MN survival, we have mainly relied upon the chick embryo activity blockade model, although to a lesser extent we have also examined the role of IGFs in normal, active embryos. For example, daily treatment of normal chick embryos *in ovo* from E6–E9 with exogenous IGF-1 promotes MN survival and also increases the number of synapses on hindlimb muscles (D'Costa *et al.*, 1998). These effects can be blocked with reagents, IGF-binding proteins (IGF-BPs), which are highly

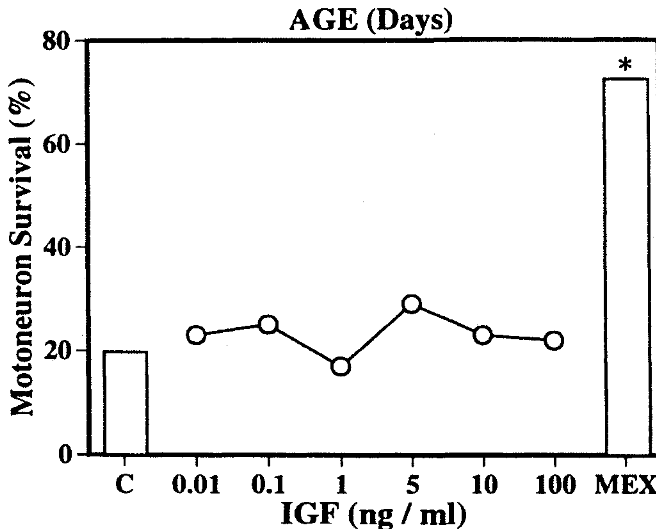

Figure 3. Survival of purified populations of chick embryo lumbar motoneurons after 48 hours *in vitro*. *C*, control; MEX, 20 μg/ml muscle extract; IGF, sum of independent groups treated with IGF-1 or IGF-2 (see D'Costa *et al.*, 1998).

specific extracellular ligands for IGFs that block the biological action of IGFs in the neuromuscular system (D'Costa *et al.*, 1998; Kiefer *et al.*, 1992; Caroni *et al.*, 1994). Because IGF-BPs are potent and specific function blocking proteins for IGFs, but lack effects on other growth/trophic factors, we have also employed them in the activity blockade model. Before describing those findings, however, it is important to point out that activity blockade also increases IGF mRNA levels in hindlimb muscles, a finding that is consistent with the access hypothesis (D'Costa *et al.*, 1998).

As summarized above, chronic activity blockade *in ovo* with curare or α-bungarotoxin (αBTX) increases intramuscular nerve branching and synapse formation and rescues MNs from PCD (Pittman and Oppenheim, 1979; Dahm and Landmesser, 1988, 1991). If these effects are regulated by endogenous IGFs, then it should be possible to inhibit them with IGF-BPs. As predicted, IGF-BP treatment *in ovo* reduces or abolishes the effects of curare-mediated activity blockade on MN survival, axon branching and synapse formation (Fig. 4). When considered together with the effects of IGF-BPs in normal, active embryos (see above), these results indicate that IGFs play a role in regulating MN survival that is consistent with the "access" hypothesis.

The mode of action of muscle-derived IGFs on the branching of developing MN axons could be either direct or indirect. A direct mode of action would argue that IGFs released from muscle act directly via IGF receptors on MNs and their axons to induce nerve branching. Increased synapse formation would follow "passively" from the permissive condition of having more nerve branches (i.e., IGF is not thought to be a synaptotrophic agent, per se). The fact that MNs express IGF receptors and that IGF promotes growth and branching of cultured MNs (Caroni and Grandes, 1990) is consistent with a direct mode of action. However, muscle activity (and inactivity) also affects muscle interstitial cells and Schwann cells at the neuromuscular junction (Caroni and Schneider, 1994; Son *et al.*, 1996) that could serve as intermediate mediators of the effects of IGFs on MNs. Additionally, despite the results of the experiments with IGF-BPs, we cannot entirely exclude the possibility that muscle-derived growth factors other than the IGFs

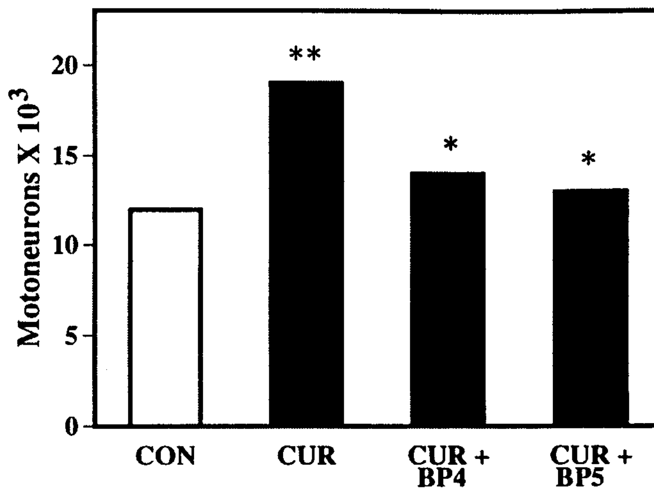

Figure 4. The number of surviving lumbar motoneurons on E10 following treatment with curare from E6 to E9 or with curare (E6–E9) plus IGF binding proteins (BP) 4 or 5 from E7 to E9. *p < 0.01 *vs* curare; **p < 0.001 *vs* CON.

may also play a role in regulating MN branching in an activity-dependent manner (e.g., see Funakoshi *et al.*, 1995; Nguyen *et al.*, 1998). Finally, even assuming that IGFs are activity-dependent, muscle-derived sprouting factors for MNs, an important remaining question is what intracellular pathways mediate the effects of IGFs on axon growth and branching. Cytoskeletal and other axonal growth associated proteins are obvious candidates (Caroni and Becker, 1992), but almost nothing is presently known about IGF actions at this level of analysis. Other candidates include cell adhesion molecules such as N-CAM which have been shown to play a role in MN axon branching (Landmesser, 1992), but the relationship, if any, of these pathways to the actions of IGFs is unknown.

5. ARE THE EFFECTS OF ACTIVITY BLOCKADE ON MN SURVIVAL CENTRALLY OR PERIPHERALLY-MEDIATED?

From the first reports 20 years ago that activity blockade in the chick embryo rescues MNs from PCD (Pittman and Oppenheim, 1978, 1979), it has been generally assumed that this effect is mediated by the classic neuromuscular blocking agents used in those experiments (e.g., Curare, α-BTX) acting at the neuromuscular junction (i.e., peripherally). However, it has recently been demonstrated that nicotinic acetylcholine receptors (nAChR) also exist on neurons in the central nervous system (Role and Berg, 1996) and that both curare and α-BTX can bind these receptors (Renshaw and Goldie, 1996) and directly reduce the activity of motoneurons, in addition to acting at the neuromuscular junction (Landmesser and Szente, 1986). Furthermore, in a recent study, Hory-Lee and Frank (1995) report that doses of curare and α-BTX that fail to reduce embryonic activity in the chick (i.e., "non-paralytic" doses) nonetheless rescue MNs and increase intra-muscular nerve branching. They also report that curare acts synergistically with muscle extract to promote MN survival *in vitro*. From these finding, Hory-Lee and Frank suggest that curare and α-BTX rescue MNs by acting at neuronal rather than muscle nAChRs (i.e., centrally *vs.* peripherally) and that this effect may be mediated by changes in calcium influx via neuronal nAChRs (e.g., see Vijayaraghavan *et al.*, 1992) rather than by changes in synaptic transmission.

Although this is an interesting hypothesis that deserves further study, there are several lines of evidence that are not consistent with this idea. First, we have not been able to confirm the claim that non-paralytic doses of curare or α-BTX promote MN survival or nerve branching (Oppenheim *et al.*, 1996). And although we have confirmed the finding that curare and muscle extract act synergistically on MN survival *in vitro*, curare alone has no effect on survival in this situation and combinations of curare and muscle extract fail to have a synergistic effect on MN survival *in vivo* (Oppenheim *et al.*, 1998). Finally, an examination of chick and mouse embryos with genetic mutations in muscle specific calcium channels that are involved in excitation-contraction coupling shows that these animals exhibit a similar phenotype to curare/αBTX-treated chick embryos, including chronic paralysis, and increased MN survival (Fig. 5), axon branching and synapse formation (Oppenheim *et al.*, 1997). Because these mutations are specific to muscle, however, and don't involve perturbations of neuronal calcium channels, these results fail to support the role of CNS mediated calcium flux via neuronal nAChRs in MN survival. However, we can't exclude the possibility that the mechanisms that mediate MN survival following activity blockade by pharmacological agents such as curare, differ from those involved in these genetic mutations.

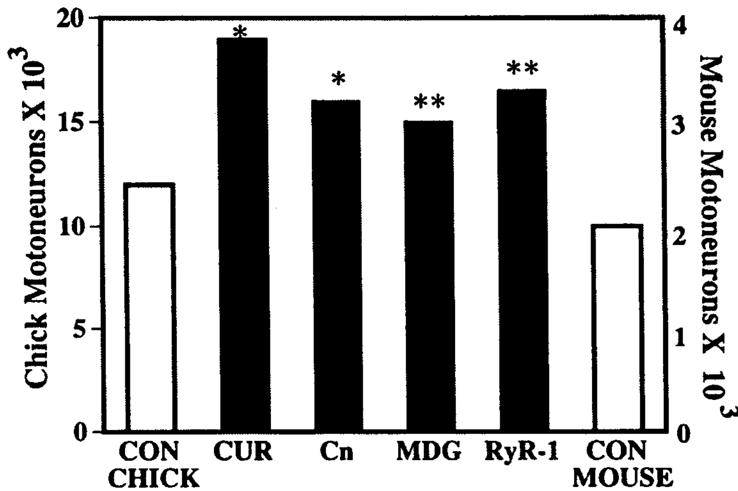

Figure 5. The number of chick and mouse embryo lumbar MNs on E10 (chick) or E18.5 (mouse). Cur, Curare E6–E9; cn, crooked neck chicken mutant; RyR-1, ryanodine receptor knock-out mouse; mdg, muscular dysgenic mouse mutant (see Oppenheim *et al.*, 1986, 1997). $^{**}p < 0.001$ *vs* con chick or mouse; $^{*}p < 0.01$ *vs* control chick.

6. SUMMARY AND CONCLUSIONS

An intriguing and puzzling feature of prenatal life is the early onset and persistence of behavior in the form of spontaneous movements and reflexes. Aside from the obvious indication that this behavior signals the onset of neuronal function, whether it has any other significance beyond this has been a question that has fascinated virtually everyone who has been privileged to observe the gymnastics of the embryo. For neurobiologists, the central issue here is whether early neuronal function is necessary either for immediate or later events in nervous system development (Oppenheim, 1981; Crair, 1999; Goodman and Shatz, 1993; Milner and Landmesser, 1999; Oppenheim and Haverkamp, 1986). It is this conceptual framework that has guided the research summarized here and which also led to the revelation that, in fact, the early activity of the embryo serves the very important role of helping to regulate neuromuscular development and, perhaps most surprisingly, helps to control the survival and maintenance of MNs.

At present the specific mechanisms by which activity regulates MN survival appear to be most easily explained by what I have called the "access" hypothesis (Oppenheim, 1989). According to this scheme, muscle activity mediated by synaptic transmission (and manifested as embryonic movement) controls the expression of a muscle-derived sprouting factor that is likely to be IGF. A counter-intuitive aspect of this hypothesis is that IGF and activity bear an inverse relationship to one another (↑ activity results in ↓ IGF, and vice-versa). IGF is thought to act via high affinity tyrosine kinase IGF-receptors present on MNs to control the growth and branching of axons which, in turn, controls MN-muscle interactions (synaptic connectivity) that ultimately determine whether MNs can get access to sufficient muscle-derived survival molecules to inhibit their own PCD. By blocking synaptic transmission, the balance in the system is biased towards increased MN survival by the up-regulation of muscle-derived IGF and increased branching, synapse formation and trophic factor access. Although many aspects of the model have been supported by experimental evidence, others remain to be confirmed making it a

model-in-progress, a kind of working hypothesis. Regardless of whether all of the details of the hypothesis are ultimately proven correct, one of the most fascinating aspects has been the apparent linkage shown by this work between neural activity and behavior, on the one hand, and some of the most fundamental aspects of nervous system development, neuronal growth and survival, on the other.

7. ACKNOWLEDGMENTS

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I

LIFELONG INFLUENCES FROM THE BEGINNING

Despite a reputation for relative isolation, both womb and egg are places where developing organisms first begin to interact with their environment. Once engaged, this early interplay between environmental factors and the genome leaves long-lasting impressions on the anatomy and behavior of organisms. The idea that organism/environment interactions guide development seems simple enough, but the stimulation received by developing organisms has some surprising and complex origins. In fact, one source of early "environmental" stimulation appears to be the behavior of the developing organism itself! For example, in the opening chapter of this book, Ronald Oppenheim (Wake Forest University) describes how the expression of prenatal reflexes and movements regulates the life and death of developing neurons and is necessary for the normal development of the nervous system. A commonly proposed mechanism for activity-dependent influences in development is one in which neurons compete for a limited quantity of some survival factor. Oppenheim has identified some of these important molecular factors and has begun to understand how they contribute to activity-dependent motoneuron survival. He shows us that multiple molecular factors can play a role in this competition for survival, and some molecules may have an "indirect" influence by initiating changes which provide the neuron with a competitive advantage for obtaining a different life-saving molecule.

A second, perhaps unexpected, source of early stimulation turns out to be spontaneous activity by sensory systems. One might assume that the influence of sensory input on the a developing organism will not be imposed until birth (or hatching), when the organism encounters the "real" environment for the first time. Not so. The chapter by Thomas Parks (University of Utah) reveals how the development of the brainstem auditory system relies on activity driven by the developing cochlea, but before sound-driven activity can be evoked to any significant extent. He has shown that this embryonic cochlear input influences multiple aspects of auditory system development, including cell survival, neurotransmitter receptor phenotype, dendritic morphology, innervation patterns, and calcium homeostatic mechanisms. Perhaps the most intriguing early influence discovered by Parks and his colleagues is that treatment with a drug which blocks glu-

tamate receptors promotes the survival of neurons, even before synapses in the auditory system are established. Thus, the influence of neurotransmitter molecules appears to be farther reaching than is often assumed. That is, their influence is not limited to when an environmental stimulus evokes their release.

Chapters by David Hill (University of Virginia) and Robert Contreras (Florida State University) add a maternal perspective to the issue of organism/environment interactions during development. Interactions between a pregnant or nursing female and her environment can in turn influence the environment experienced by her offspring. These early experiences, transmitted from the mother via the uterine environment or milk, can have long-term effects on behavior, physiology, and neuroanatomical structure. These chapters show how restricted (Hill) or elevated (Contreras) perinatal salt exposure alters adult salt preference, sensory and behavioral sensitivity to salt, blood pressure, and the morphology of salt-sensitive pathways to the brain. Here again, the work challenges some commonly held assumptions on how deprivation alters development. First, although the salt intake is manipulated, and some of the effects are specific to sodium transduction, it appears that the sensory aspect of the manipulation is, at least in some cases, irrelevant. Rather, the effects of this manipulation may emerge through systemic effects on developing hormonal systems. Second, we see once again that early events can influence a sensory system even before the system is functional. In fact, Hill shows that dietary sodium restriction has effects on neurons in the gustatory system even when the restriction takes place before the neurons are born!

2

COCHLEAR INFLUENCES ON DEVELOPMENT OF THE BRAINSTEM AUDITORY SYSTEM

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1. STUDYING THE EAR'S INFLUENCE ON THE DEVELOPING BRAIN

1.1. Introduction

Experimental work from several laboratories, including that reviewed by Hyson and Sanes in this volume, has shown that from the time cochlear nerve synapses are first formed on CNS auditory neurons, the developing ear exerts a powerful influence on the developing brain (see also Moore, 1992). My laboratory has studied how influences from the ear affect survival, form, connectivity, calcium homeostasis mechanisms, and neurotransmitter receptor properties of chick brainstem auditory neurons.

1.2. Manipulating the Developing Chick Auditory System

Because of the many similarities between the auditory systems of birds and mammals and the many advantages of using avian embryos in developmental studies (Rubel and Parks, 1988), we have used a chick embryo model to study the normal development of brainstem auditory circuits and the effects of early hearing loss on this development (Fig. 1). Hearing loss has been produced by surgical destruction of the otocyst or cochlea or by the use of earplugs (Parks, 1997). The otocyst is the embryonic precursor of the inner ear and acousticovestibular nerve; since cochlear nerve axons do not enter the brain until embryonic day (E) 4 and the cochlea is not functional until about E11 (Rubel and Parks, 1988), surgical removal of the otocyst (unilaterally or bilaterally) on E3 results in the auditory CNS developing without ever receiving synaptic input from the ear (Parks, 1979; see Fig. 2). In some experiments, the cochlea has been surgically destroyed in chickens after hatching. Earplugs formed by injecting liquid plastic hearing aid sealer into the external auditory meatus of E18 chick embryos have also been used to produce a severe (40–50 dB) conductive hearing loss combined with some sensorineural

The Biology of Early Influences, edited by Hyson and Johnson
Kluwer Academic / Plenum Publishers, New York, 1999.

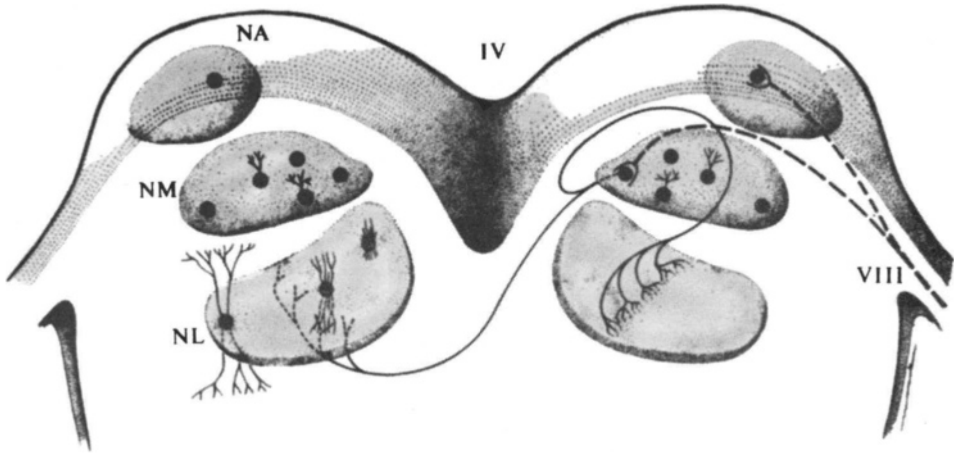

Figure 1. Schematic representation of chick brain stem auditory anatomy. Cochlear division of the eighth cranial nerve (VIII) provides tonotopic innervation of nucleus angularis (NA) and nucleus magnocellularis (NM), forming large calyx-like axonal endings in NM. Each NM neuron sends one axon branch to dorsal dendrites of neurons in ipsilateral nucleus laminaris (NL), and another branch to ventral dendrites of contralateral NL. Axon terminal fields of NM neurons are oriented perpendicularly to the axis of tonotopic organization, which extends from posterolateral (representing low-frequency sounds) to anteromedial (high frequencies). This axis also defines gradients in NL dendritic length and number; cells have shorter but more numerous primary dendrites anteromedially than caudolaterally. IV, fourth ventricle.

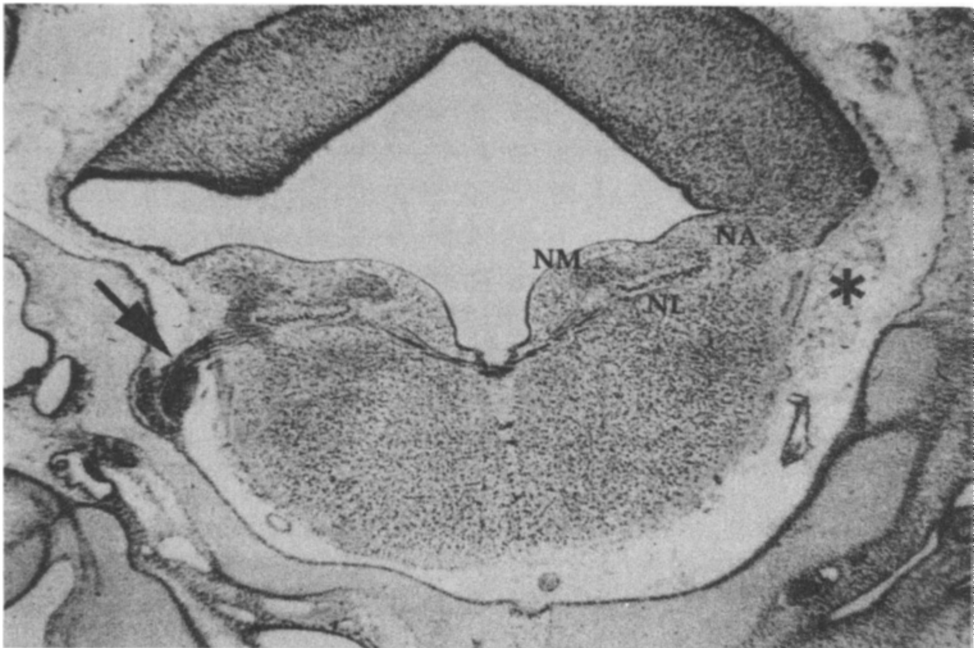

Figure 2. Photomicrograph of protargol-stained coronal section through the skull and brain stem of E9 chick embryo from which the right otocyst had been removed surgically at E3. The eighth nerve appears normal on unoperated left side (arrow), but is absent on the right (*). Brain stem auditory nuclei (NA, NL, and NM) are present bilaterally.

loss; the CNS degenerative changes associated with earplugs result from the sensorineural component (Tucci and Rubel, 1985).

2. NEURON SURVIVAL

2.1. Effects of Early Cochlear Damage

Otocyst removal at E3 causes no observable effects on development of the cochlear nuclei angularis (NA) and magnocellularis (NM) or of nucleus laminaris (NL; comparable to the medial superior olivary nucleus of mammals) until about E11, when the cochlea and cochlear nerve synapses are becoming functional (Jackson *et al.*, 1982). At this time, some neurons in the NM and NA (but not the NL) that would normally survive begin to die, leading to deficits in neuron number of about 33% in NM and 40% in the NA by E17 (Parks, 1979; Fig. 3). There is little or no normal neuronal death in NM, a small amount of normal death in NA, and 19% normal neuronal loss in NL. After otocyst removal, neurons in NA and NM also fail to increase in size normally, with the result that the affected neurons are about 20% smaller than controls. Also, in the absence of cochlear nerve input during development, a portion of the NA migrates between E9 and E19 to an ectopic ventromedial position (Parks, 1979). The death of a substantial proportion of neurons in the chick cochlear nuclei after early deafferentation was one of

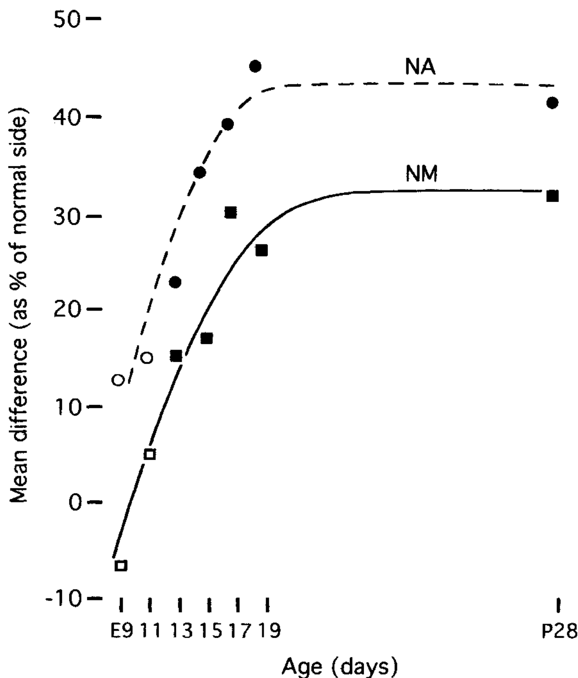

Figure 3. Effect of unilateral removal of otocyst on the number of neurons in NA and NM between E9 and P28. Each symbol represents the mean of precentage differences obtained by comparing neuronal number on deafferented and normal sides of the brain in 3 animals at that age. Statistically significant decreases in neuronal number in both nuclei (filled symbols) are evident only after E11, when the cochlear nerve normally forms functional synapses there.

the first examples of afferent control over neuron survival (Levi-Montalcini, 1949) and remains one of the best. The finding that cochlea removal in hatchling chickens produces similar loss of neurons in NM (Rubel *et al.*, 1990) has allowed study of the physiological and biochemical mechanisms involved in afferent-dependent neuron survival; as discussed by Hyson [this book], a major focus of this work has been glutamate receptors.

2.2. Prevention of Neuronal Death by Glutamate Receptor Antagonists

At least three major subtypes of glutamate receptor (AMPA, NMDA and metabotropic) appear to be present on developing chick brainstem auditory neurons (Zhou and Parks, 1992; Zirpel *et al.*, 1998) and it is a major focus of research in this laboratory to understand how activation of these receptors at different periods in development affects NM and NL neurons. Because potent and selective antagonists of AMPA and NMDA receptors are available, we used the *in ovo* drug administration method (see Oppenheim, this book) to study ionotropic glutamate receptor contributions to cell survival in the brainstem auditory nuclei (Solum *et al.*, 1997). As mentioned above, about 20% of NL neurons undergo programmed cell death during normal development. The competitive AMPA/kainate receptor antagonist CNQX, when administered at doses of 200–300 μg/day from E8 to E15, prevented programmed neuronal death in NL through at least posthatching day (P) 8, without producing obvious anatomical or behavioral abnormalities. CPP, a potent and specific antagonist of NMDA receptors, had no effect on normal cell death in NL, even at doses that affect neuronal survival in the chick spinal cord (Fig. 4). Treatment with CNQX or the more highly selective NBQX from E8 to E10, before the onset of synaptic transmission in NM and NL, was also effective in preventing normal neuronal death in NL (Fig. 4). CNQX, given from E8 to E15 or only from E8 to E10, also blocked the 33% neuronal loss in the NM that follows ipsilateral otocyst removal (Fig. 4). Thus, we concluded that blockade of AMPA receptors, but not NMDA receptors, can completely and permanently prevent both normal programmed cell death in NL and deafferentation-induced death in NM (Solum *et al.*, 1997).

Understanding how AMPA receptor antagonists promote neuronal survival in NL and NM is of considerable interest since it is thought to be the first example of AMPA receptors having such an important influence on cell survival during development. Because AMPA receptors mediate rapid synaptic transmission between the cochlear nerve and NM (Zhou and Parks, 1992; Zhang and Trussell, 1994) and between NM and NL (Zhou and Parks, 1991), our initial hypothesis was that AMPA antagonists would act by interfering directly with the rhythmic spontaneous activity generated in the inner ear and transmitted synaptically to NM and NL during development (Lippe, 1994). Analysis of the effects of CNQX and NBQX on spontaneous embryonic motility (which had previously been reported to be mediated by glutamate receptors in the spinal cord) at E10 showed that the doses effective in preventing neuronal death suppressed motility for less than 8 hours (Solum *et al.*, 1997). This suggested that AMPA antagonists given on E10 would not be effective in suppressing synaptic transmission in the auditory nuclei on E11, when normal synaptic function begins. Thus, we concluded that the survival-promoting effects of AMPA antagonists given from E8–10 were most likely not produced directly via AMPA receptor-mediated electrical activity in NM and NL. Rather, we suggested that these drugs might permanently affect neuronal physiology by binding to receptors prior to the onset of normal synaptic function or by increasing production of a diffusible survival-promoting factor.

The supposition that the observed survival-promoting effects of AMPA antagonists

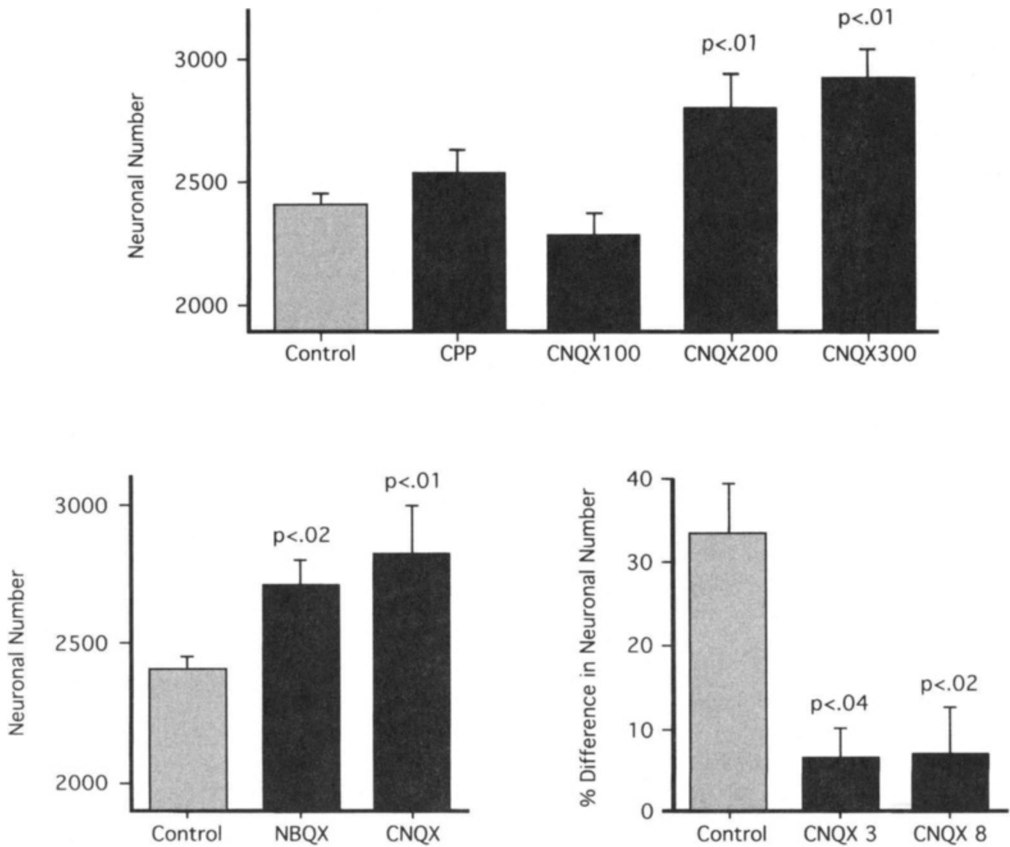

Figure 4. Antagonism of normal and deafferentation-induced neuronal death by *in ovo* treatment with AMPA receptor antagonists. **Top**, Antagonism of AMPA/kainate receptors, but not NMDA receptors, reduces normal neuronal death in nucleus laminaris (NL). Chick embryos were treated from E8–E15 with 200 µl/day of saline or vehicle containing the AMPA receptor antagonist CNQX or the NMDA receptor antagonist CPP and cell number was determined at E17. Bar graphs show means ± SE for NL neuron number at E17. At doses up to 400 µg/day, CPP had no reliable effect. CNQX produced a dose-dependent increase in NL neuron number above control values, with statistically significant effects seen at 200 and 300 µg/day. **Lower left**, Treatment from E8 to E10 with CNQX or NBQX (a more potent and selective AMPA receptor antagonist) results in significant increases in neuronal number in NL at E17. Bar graphs show means ± SE for NL neuron number in animals treated with vehicle only, 200 µg/day CNQX, and 100–150 µg/day NBQX. The drug-treated groups had significantly more NL neurons than the control group but did not differ reliably from each other. **Lower right**, CNQX treatment from E8 for 3 or 8 days prevents deafferentation-induced neuronal death in nucleus magnocellularis (NM). Bar graphs show means ± SE for percent differences between NM neuron number on the operated (O) and unoperated (U) sides of animals that received unilateral removal of the otocyst on E3; percent difference is calculated as [1-(O/U)] × 100. Control animals (saline only) showed a loss of one-third of the neurons on the opeated side of NM, whereas treatment with 200 µg/day CNQX resulted in statistically significant reductions in neuronal loss compared with controls.

could not be due to activity-dependent effects directly on NM and NL was undermined by the subsequent report of Chub and O'Donovan (1998), who monitored synaptic function and motility in chick spinal cords exposed to glutamate, GABA or glycine receptor antagonists. These authors showed that although glutamate receptor blockade suppresses motility temporarily (with a time-course similar to that observed in our study), the recovery of motility occurs via GABAergic and glycinergic circuits that are somehow able to

effect excitation of spinal motor neurons while glutamate receptors are still fully blocked. In view of these results, it must be concluded that the mechanism by which AMPA receptor blockade protects brainstem auditory neurons from normal or deafferentation-induced death during development is unknown.

We are currently studying the effects of AMPA antagonists on normal developmental death of cochlear ganglion neurons. There is reason to believe that these cells will be more amenable than CNS auditory neurons to an *in vitro* analysis of the sort used so productively by Oppenheim (this volume) to study the signals controlling survival of spinal motor neurons. The unusually potent influence that AMPA receptors have on the development of auditory neurons has prompted examination of the molecular structure and functional properties of these receptors.

3. THE AMPA RECEPTOR OF AUDITORY NEURONS

3.1. Structure and Function of "Auditory" AMPA Receptors

AMPA receptors are assembled from four protein subunits, termed GluRs 1–4 (Bettler, 1995). Native AMPA receptors include a variety of subunit, splice variant, and mRNA editing combinations which result in the diverse functional properties that underlie specialized information-processing features of different neurons (Michaelis, 1998). Auditory neurons show a number of striking morphological and functional specializations that can be related to their roles in hearing (Trussell, 1997; Oertel, 1997); not surprisingly, the AMPA receptors expressed by auditory neurons in birds and mammals are also highly specialized. In the chick brainstem, auditory neurons have the fastest desensitization kinetics of any AMPA-activated receptor—almost five-fold faster than those expressed by brainstem motor neurons, for example (Raman *et al.*, 1994). The neurons of NM, NA and NL also show very strong agonist-evoked cobalt uptake via calcium-permeable AMPA receptors (Zhou *et al.*, 1995; Otis *et al.*, 1995); as shown in Fig. 5, this permeability to divalent cations is developmentally-regulated.

We have used mRNA analysis, whole-cell patch-clamp recordings, immunohistochemistry, and agonist-evoked cobalt uptake to compare the molecular and functional properties of AMPA receptors in auditory neurons of the chick cochlear ganglion, NM and NL with those of motor neurons in the glossopharyngeal/vagal nucleus from E10 through hatching. There is a characteristic "auditory" AMPA receptor that is distinguished from the motor neuron receptor by (in addition to faster desensitization

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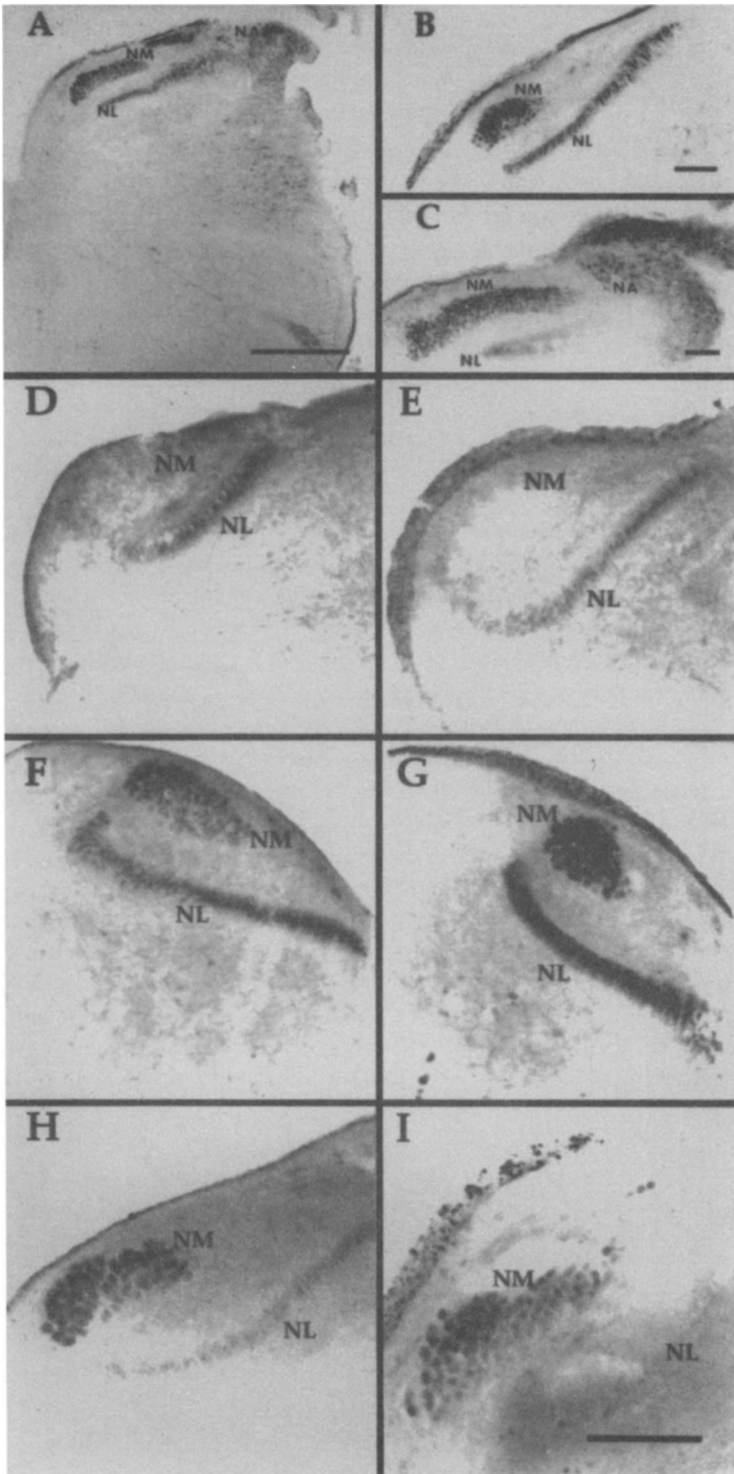
Figure 5. Kainate-evoked cobalt uptake in the chick brain stem auditory nuclei. **A**, low-power view of the right side of the brain stem at E15 exposed to 100 μM kainate; heavy staining is seen in the nuclei magnocellularis (NM), laminaris (NL) and angularis (NA) but only scattered cobalt-positive neurons are seen elsewhere in the brain stem. Calibration bar = 0.5 mm. **B**, a section through the rostral NM and NL showing heavy labeling of individual neurons. C, a section through NA and the posterior NM and NL at E15 showing heavily stained large neurons scattered throughout NA and heavy staining in NM and NL. Calibration bar for **B** and **C** = 0.1 mm. **D–I** show that the pattern and intensity of cobalt uptake in the NM and NL evoked by 30 μM kainate change markedly during embyronic development. At embryonic days (E)9 (**D**) and E11 (**E**), kainate evokes cobalt accumulation only in NL. By E13 (**F**), there is moderately strong staining in both NM and NL and at E15 (**G**) there is strong cobalt uptake in both nuclei. At E18 (**H**), kainate evokes only weak staining in NL and moderate staining in NM, with a few NM neurons showing stronger staining. By E21 (**I**), no cobalt uptake is seen in NL and staining is moderate to weak in NM, with the exception of a few strongly-stained neurons. Calibration bar for **D–I** = 0.2 mm.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kinetics) lower relative levels of GluR2 flip, higher relative levels of GluRs 3 and 4 flop, lower R/G editing of GluRs 2–4, higher relative expression of the novel GluR4 c-terminal splice variants described by Ravindranathan *et al.* (1997), greater permeability to divalent cations, predominantly inwardly rectifying current/voltage (I/V) relationships, and greater susceptibility to block by Joro spider toxin, a specific noncompetitive blocker of calcium-permeable glutamate receptor channels (Ravindranathan *et al.*, 1999).

3.2. Cochlear Input Is Required for Maturation of NM AMPA Receptors

Zhou and Parks (1992) used pharmacophysiologic methods and brain slice preparations to study the normal development of glutamatergic synaptic transmission between the cochlear nerve and NM. We found that the sensitivity of synaptic transmission to blockade by NBQX declined by a factor of 10 between E14 and E21 (i.e., the half-maximal inhibitory concentration [IC_{50}] for NBQX increased from 0.27 to 2.7 μM over this period). When similar methods were used to study aberrant glutamatergic NM-to-NM synapses in animals with unilateral otocyst removal (see section 4.2 below), Zhou and Parks (1993) found that the immature pattern of greater sensitivity to NBQX was maintained at E21 and for at least 2 weeks after hatching (Fig. 6). The failure of the aberrant synapses to undergo normal maturation of NBQX sensitivity suggests that the cochlear nerve normally supplies some influence necessary for maturation of the AMPA receptors in NM. Developmental comparisons of AMPA receptor mRNAs from deafferented and normally innervated NM neurons may reveal a structural basis for the maturation of NBQX sensitivity. This should facilitate development of an assay to identify cochlear nerve-related influences associated with that maturation.

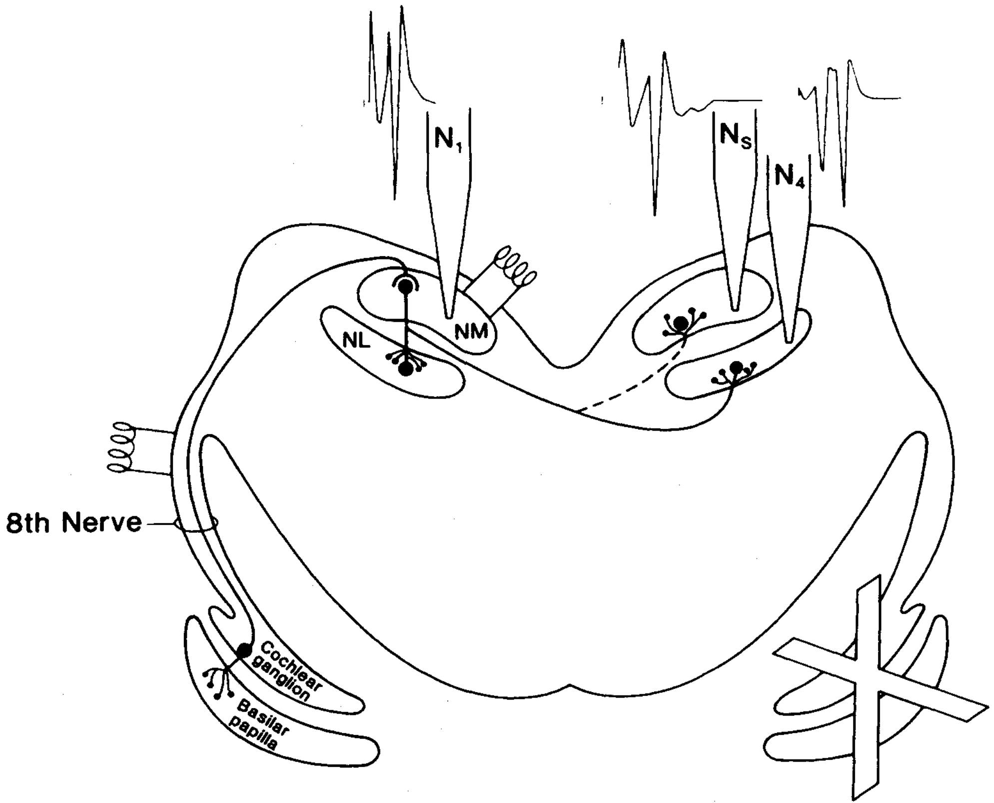
4. NEURONAL FORM AND CONNECTIVITY

4.1. Characteristic Shapes of Brainstem Auditory Neurons

The unusual shapes of neurons in the brainstem auditory nuclei have been shown in several cases to be closely related to functional specializations required for the processing of auditory information (Oertel, 1997; Trussell, 1997). The adendritic or parvodendritic neurons of NM achieve their simple mature form through a complex series

▶

Figure 6. The aberrant NM-to-NM synapses maintain pharmacologically-immature AMPA receptors. **Top**, a schematic diagram of a coronal section through the chick brainstem auditory system showing the experimental preparation used in these experiments. The "X" on the right basilar papilla indicates previous otocyst removal and the dotted line on the right indicates the aberrant NM-to-NM pathway induced by otocyst removal. By electrically stimulating the left cochlear (8th) nerve, it is possible to record a multi-component field potential (N_1; illustrated next to the recording electrode in NM) in the ipsilateral NM. Electrical stimulation of the left NM allows recording of multi-component field potentials from the right NM via the aberrant pathway (the N_s) and an N_4 representing normal synaptic currents in nucleus laminaris (NL). Drug-induced changes in the amplitude of the field potentials were used to construct concentration-response curves for NBQX, from which IC_{50} values are determined by logistic regression. **Bottom**, graph summarizes developmental changes in the potency of the AMPA receptor antagonist NBQX in inhibiting synaptic transmission at normal cochlear nerve synapses in NM (N_1) and at aberrant NM synapses formed in the contralateral NM after otocyst removal (N_s). The half-maximal inhibitory concentration (IC_{50}) of NBQX on N_1 increases about 13-fold between E14 and P14, but aberrant synapses maintain a low IC_{50} and show no change through 2 weeks after hatching. Each point represents the mean of three or more independent experiments.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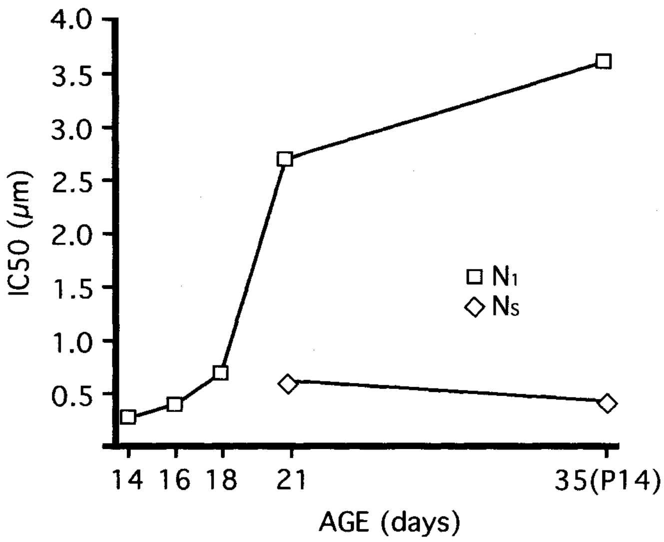
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of morphological transformations (Jhaveri and Morest, 1982). Between E11 and E15, along a rostromedial-to-caudolateral gradient, multipolar NM neurons lose all of their dendrites to become unipolar; on average each NM neuron loses 10 dendrites (Parks and Jackson, 1984). At the same time that their NM targets are undergoing these changes, cochlear nerve axons undergo a dramatic decrease in terminal branching and, thereby, reduce the number of NM neurons with which each axon makes synaptic contact (Jackson and Parks, 1982). Surprisingly, otocyst removal, despite leading to the death of one-third of NM neurons and shrinkage of the survivors, has no effect on the extent, timing or spatial gradient of dendritic loss in the NM (Parks and Jackson, 1984; Fig. 7).

An unusual feature of development in NM is that after losing all of their primary dendrites by E15 as described above, 40% of these neurons sprout a single new "permanent" dendrite about E18 that grows during the next few weeks. A mixed conductive-sensorineural hearing loss produced unilaterally from E18 with plastic earplugs does not affect the proportion of NM neurons that grow a permanent dendrite but does retard

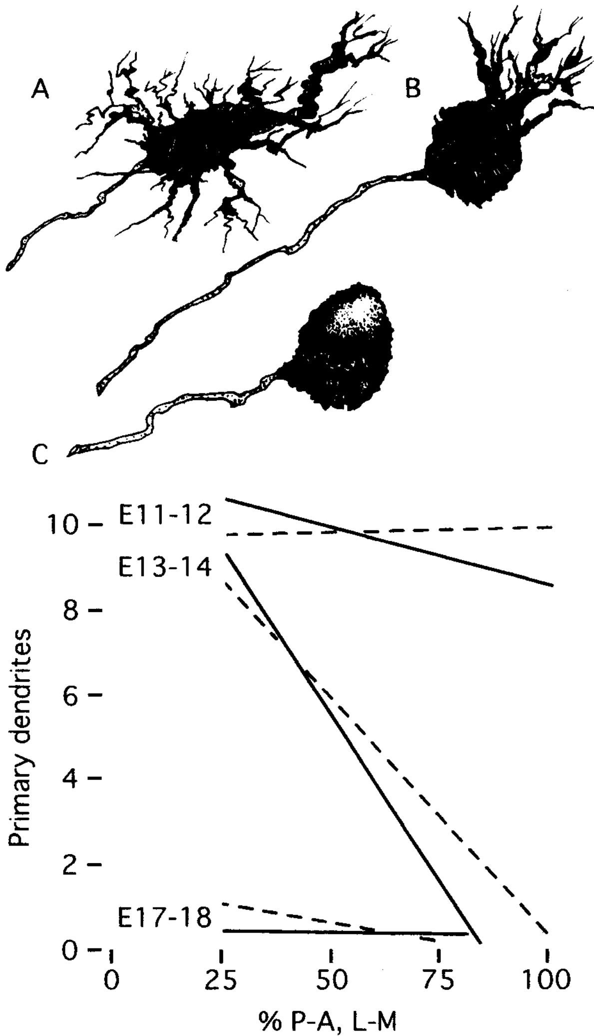


Figure 7. Illustrations of the spatiotemporal gradient of dendritic loss during development of NM and the independence of this process from cochlear influences. **A–C**, stylized drawings of horseradish peroxidase-stained NM neurons showing normal morphologic transformation from multipolar to unipolar neurons. **D**, P-A—posterior-to-anterior, L-M—lateral-to-medial. At E11–12 (**A**), neurons throughout NM have numerous (about 10 on average) dendrites; these are rapidly lost between E13 and E16 along an anteromedial-to-posterolateral gradient withihn NM. At E14, neurons about midway along this axis have only a few dendrites (**B**), whereas more anterior cells (**C**) have lost all of their dendrites. By E16–17, all neurons in NM resemble the neuron in C. **D**, The graph consists of six multiple linear regression lines relating the number of primary dendrites to the position of neurons within NM at E11–12, E13–14, and E17–18. Data are from the deafferented right side of the brain (dashed lines) and normally innervated left side (solid lines). There were no significant differences between the deafferented and normal sides.

the posthatching growth of these dendrites (Conlee and Parks, 1983), as well as the NM cell bodies (Conlee and Parks, 1981) (Fig. 8).

The bipolar neurons of the NL receive massive excitatory synaptic input to their dorsal dendritic tufts from the ipsilateral NM and to their ventral dendritic tufts from the contralateral NM. Agmon-Snir *et al.* (1998) have recently shown that this gradient of dendritic structure enriches the computational power of NL neurons and thereby improves their performance as coincidence detectors in the neural circuit subserving low-frequency sound localization. Beginning about E14, and continuing for some weeks after hatching, NL neurons undergo a complex series of morphologic transformations that result in the formation of a steep rostromedial-to-caudolateral gradient of increasing total dendritic length across the nucleus (Smith, 1981). Since this gradient parallels the tonotopic axis of the NL, it was hypothesized that acoustic experience might have some role in shaping the dendritic gradient. This hypothesis was tested in experiments in which one or both otocysts were removed at E3 and dendritic structure was quantified in Golgi-stained neurons from E17 neurons (Fig. 9). Parks (1981) found that unilateral otocyst removal results in a mean 44% decrease in the length of NL dendrites connected to the deafferented NM, but did not significantly affect the spatial gradient of dendritic length. Parks *et al.* (1987) found that bilateral otocyst removal had a much less severe effect than unilateral deafening on dendritic length; even this bilateral deafening, however, permitted development of relatively normal patterns of dendritic organization, including the

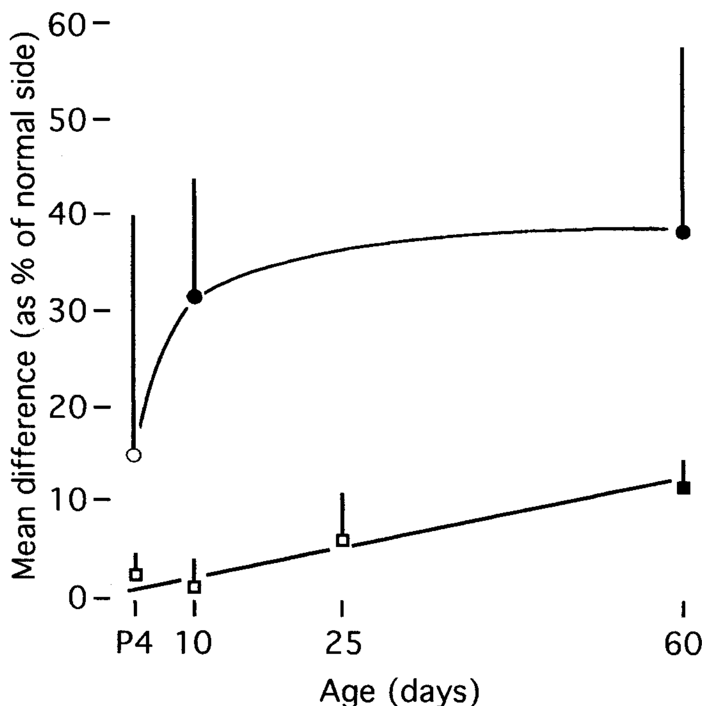


Figure 8. Effects of severe unilateral mixed conductive and sensorineural hearing loss initiated at E18 on development of neuronal cross-sectional area (squares) and total dendritic length per cell (circles) in NM. Each symbol represents the mean ± SE of deprived versus normal percentage differences calculated for eachof 4–9 animals. Hearing loss retards the growth of NM neurons, producing statistically significant differences (filled symbols) in dendritic length by P10 and in cell body size by P60.

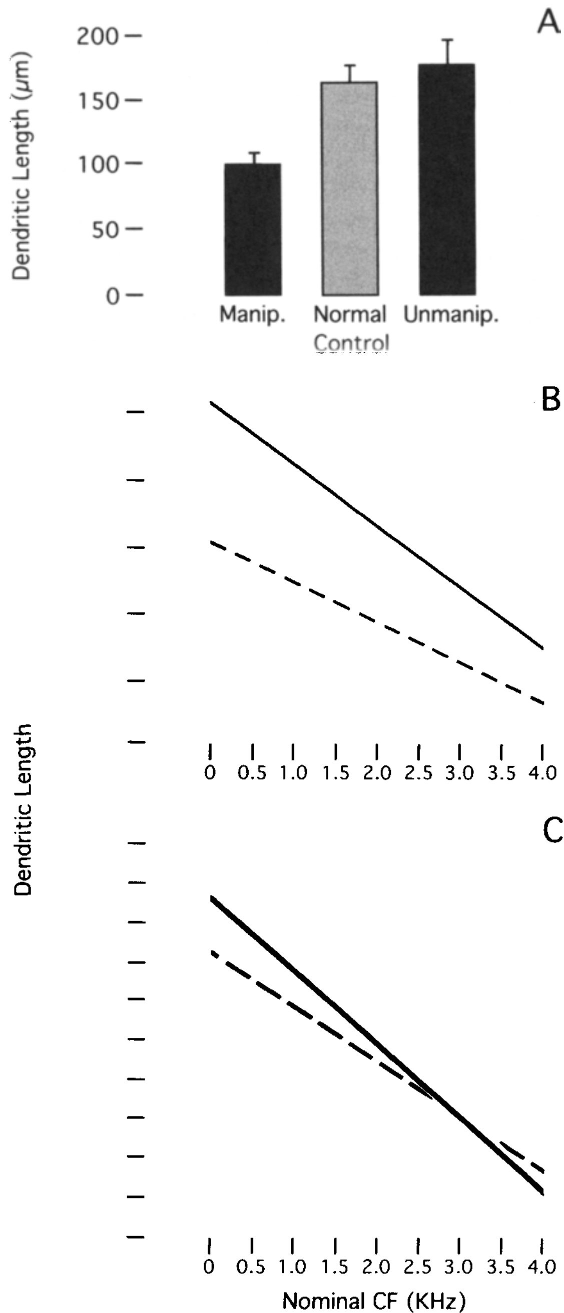


Figure 9. Effects of otocyst removal at E3 on dendritic structure in nucleus laminaris (NL) at E17. **A**, measurements of dendritic length in animals with removal of the right otocyst show that the "manipulated" dendrites (i.e., the dorsal dendrites of the right NL and ventral dendrites of the left NL) are a statistically-reliable 44% shorter than the "unmanipulated" dendrites on the same neurons or NL dendrites in unoperated controls. Nevertheless, as shown in **B**, the spatial gradient of dendritic length in both manipulated (dashed line) and unmanipulated NL dendrites is not significantly different. These lines represent the multiple linear regression of dendritic length on position within the nucleus (expressed as the nominal characteristic frequency, CF). The lowest CFs are found posterolaterally in NM and the highest CFs (to 4 kHz) anteromedially. The regression lines in **C** show the relationship between dendritic length and position in NL for normal controls (solid line) and bilateral otocyst (dashed line) animals studied at E17. Total dendritic length is highest posterolaterally (low CF) in both groups, and slopes of the two regression lines are not significantly different, demonstrating that spatial gradients of dendritic structure in NL can develop independently of cochlear input.

rostromedial-to-caudolateral gradient of increasing dendritic length (Fig. 9). We concluded that although attainment of normal dendritic size in the NL is dependent upon a normal inner ear, most of the highly specialized dendritic organization of this nucleus can develop normally in the complete absence of peripheral influences. These results also suggested that each brainstem auditory nucleus is organized along an intrinsically determined axis that specifies how incoming auditory afferent axons will be organized into tonotopic arrays.

4.2. Appearance of a Novel Pathway After Otocyst Removal

To our surprise, we found that otocyst removal induces formation of an aberrant axonal projection to the ipsilateral NM from the contralateral NM; in the case of bilateral otocyst removal, each NM innervates the other. The aberrant fibers arise from the contralaterally directed branch of each NM axon near the medial border of the contralateral NL, are present in the NM by E7, form functional glutamatergic synapses, and persist after hatching (Jackson and Parks, 1988). The aberrant NM axons go only to the contralateral NM (and not, for example, to adjacent vestibular nuclei that have also been deafferented by otocyst removal) and form a remarkably normal tonotopic array in their new target (Lippe *et al.*, 1992). Although the aberrant NM-to-NM axons retain the boutonal form and size typical of NM axon terminals in the NL (rather than assuming the large calyx-like form of cochlear nerve terminals in the NM), the aberrant endings are deeply invaginated by their NM targets and form "hybrid" contacts with features of both normal NM-NL endings and cochlear nerve-NM endings (Parks *et al.*, 1990; Parks and Taylor, 1993; Fig. 10). There is good evidence that no aberrant axons other than those from the contralateral NM are present in the NM after otocyst removal (Parks *et al.*, 1990). Although it was attractive to speculate that this aberrant projection could account for the survival of two-thirds of the NM's neurons after otocyst removal, experiments in which the left otocyst and right rhombic lip (precursor of the NA, NM and NL and other structures) were surgically destroyed showed that the left NM survives with little apparent reduction in neuronal number (unpublished observations).

5. CALCIUM HOMEOSTASIS IN AUDITORY NEURONS

5.1. Development of Calcium Homeostasis in the Nucleus Magnocellularis

Neurons in NM require cochlear nerve activation of metabotropic glutamate receptors (mGluRs) to maintain intracellular free calcium concentrations $[Ca^{2+}]_i$. Interrupting this activation results in an increase in $[Ca^{2+}]_i$ followed by atrophy, degeneration and death of many NM cells (Zirpel *et al.*, 1995). Brainstem auditory neurons experience increasing levels of spontaneous and stimulus-driven electrical activity during development; mature auditory neurons are some of the most electrically and metabolically active cells in the CNS. Synaptic depolarization can lead to calcium influx via both ligand-gated channels (e.g., AMPA and NMDA receptors) and voltage-gated channels, as well as liberation of internal calcium stores via various activity-dependent mechanisms (e.g., via mGluR-coupled release of inositol trisphosphate, IP_3). Calcium ions are an important intracellular messenger and the concentration of free calcium ($[Ca^{2+}]_i$) must be maintained within the narrow normal range to avoid toxicity. We have been interested in learning how brainstem auditory neurons maintain calcium homeostasis in the face of steadily increasing afferent excitation during development.

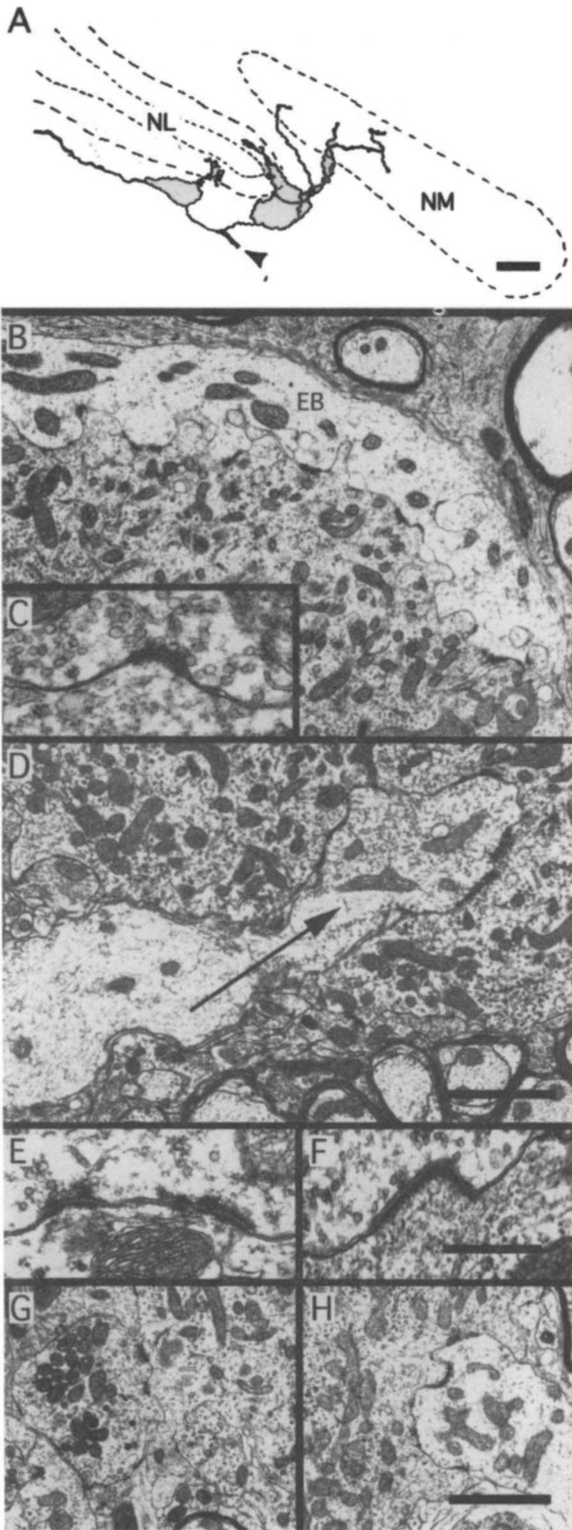


Figure 10. Morphology of the aberrant NM-to-NM synapses formed after otocyst removal. **Top**, camera lucida drawing of a single HRP-stained NM axon in an E17 animal which had received an ipsilateral otocyst removal at E3. The labeled axon (which originates from an NM neuron on the contralateral side of the brain) enters the section (arrowhead) ventral and medial to nucleus laminaris (NL). Dorsal is up and lateral to the left. A main axon branch continues laterally, giving off normal vertically-directed branches that end in sprays of boutons on ventral dendrites of NL. Just lateral to the arrowhead, another branch ascends, giving off branches with terminal boutons to the dorsal side of NL and a large branch that ascends to NM and forms boutonal terminals there. Scale bar = 50 μm. **B–H** are electron micrographs allowing comparison of the morphologies of axo-somatic endings formed normally by the cochlear nerve in NM and by NM in NL, and the aberrant NM-to-NM projection. **B**, a calyciform endbulb (EB) formed by a cochlear nerve axon on a NM neuron. **C**, a synaptic profile from a cochlear nerve ending, showing a typical postsynaptic density and no apparent presynaptic dense material. **D**, an aberrant NM axon (arrow) can be seen invaginating its NM target neuron and forming a boutonal ending with multiple active zones. **E**, synapse associated with a normal NM ending in nucleus laminaris, such as that shown in **G**; note the prominent pre- and postsynaptic densities. **F**, synapse associated with aberrant NM-to-NM axonal endings, such as that shown in **H**; note the prominent postsynaptic density and the absence of presynaptic dense material. Scale bars = 2 μm for **B, G** and **H,** 1 μm for **D,** and 0.5 μm for **C**, **E** and **F**.

Since the main components of the neuronal calcium-regulating mechanism are well known, we (Zirpel *et al.*, 1998) carried out a developmental survey of calcium homeostasis in NM using immunohistochemistry and quantitative Western blotting to assess changes in expression of group I mGluRs (i.e., mGluRs 1 and 5), inositol trisphosphate receptor (IP_3R), and sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs). In parallel with these biochemical studies, we studied developmental changes in calcium homeostatic physiology with fura-2 imaging of agonist-evoked $[Ca^{2+}]_i$ increases in NM in brain slices and confocal laser scanning microscopic analysis of individual fluo3-labeled NM neuron responses to agonist-evoked calcium influxes. This study has shown that calcium homeostasis becomes activity-dependent with the increasing synaptic activity and maturation that occurs after the onset of synaptic function at E11. During the course of development, group I mGluR expression peaks at the beginning of spontaneous activity (E13) and then declines sharply. Group I mGluR release of Ca^{2+} from intracellular stores increases with increasing synaptic maturation, perhaps due to increasing expression of IP_3R. SERCA expression decreases with increasing synaptic maturation and at E13 there is an associated decrease in NM neurons' ability to recover from a Ca^{2+} signal. Before high levels of spontaneous activity occur, NM neurons show faster increases in $[Ca^{2+}]_i$ and slower buffering kinetics, resulting in a larger integrated Ca^{2+} signal. This phenomenon disappears with increasing synaptic activity and maturation. We concluded that mGluR-mediated calcium homeostasis in NM develops in parallel with synaptic activity (Zirpel *et al.*, 1998).

5.2. Development and Activity-Dependent Regulation of Calretinin Expression

One important element of the neuronal calcium homeostasis machinery not studied by Zirpel *et al.* (1998) is the "EF-hand" family of neuron-specific calcium buffering proteins: calretinin (CR), calbindin and parvalbumin. Although mammalian auditory neurons frequently express more than one of these proteins, the chick NA, NM and NL express only CR (Rogers, 1989). Since CR appears likely to be an important contributor to auditory neurons' calcium homeostasis, we examined the normal development of CR expression and the effects on this development of manipulating synaptic input and excitation. By means of *in situ* hybridization and immunohistochemistry, we found that CR expression is very low prior to E11 and increases rapidly after that age to the very high levels characteristic of the mature auditory system (Fig. 11). Although the onset of CR expression is temporally correlated with the onset of synaptic activity, removal of one or both otocysts had no effect on CR expression nor did middle ear ossicle removal (Parks *et al.*, 1997a). We concluded that there is an activity-independent level of expression of CR in the brainstem auditory nuclei. In subsequent *in vitro* brain slice experiments, we found that expression above this constitutive level can be regulated by synaptic activity. As shown in Fig. 12, activation of AMPA receptors on NM neurons by kainic acid or electrical stimulation of the cochlear nerve resulted in higher levels of CR expression (Parks *et al.*, 1997b) that could be blocked by CNQX.

6. CONCLUSIONS

The studies reviewed above have identified a number of features of brainstem auditory system development that occur independently of cochlear influences and others that

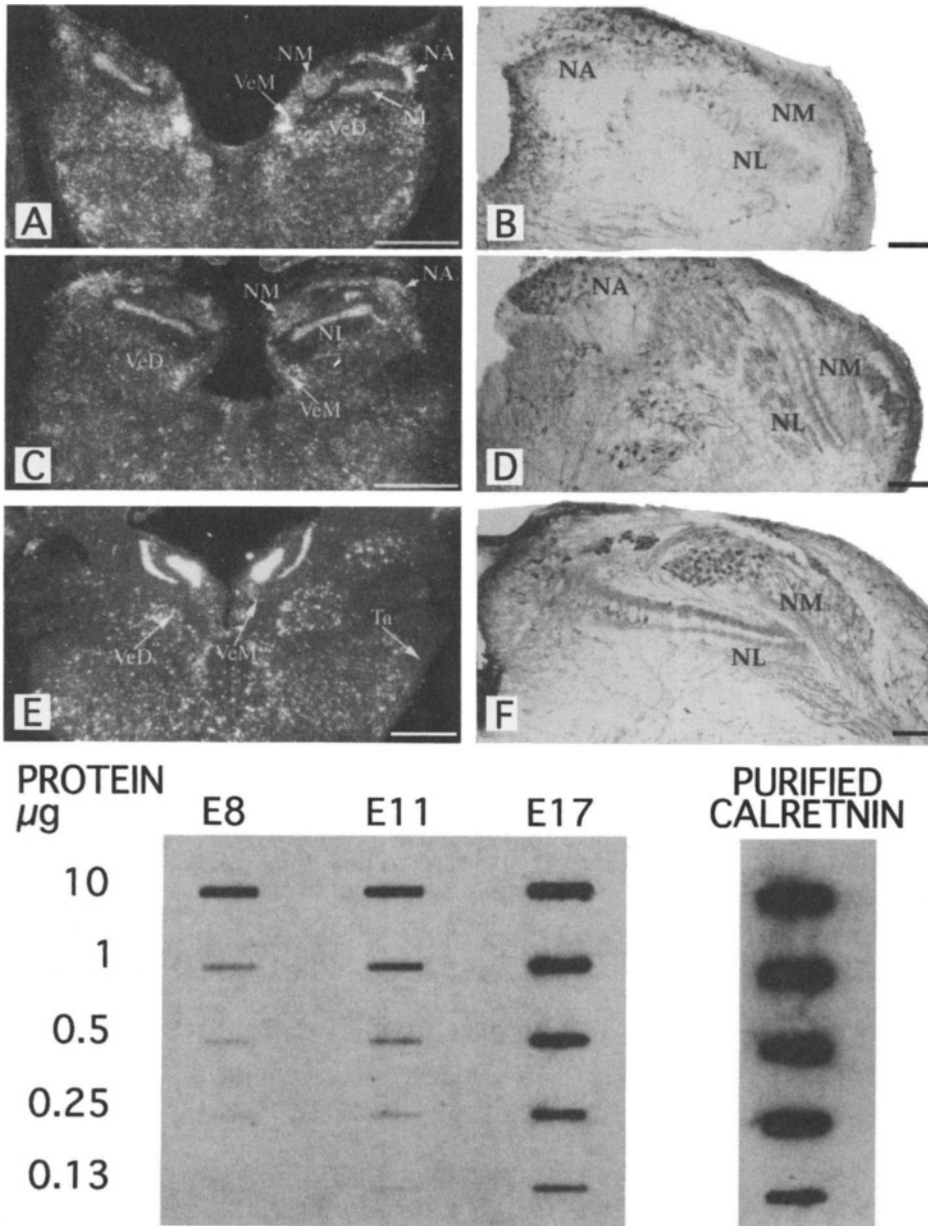
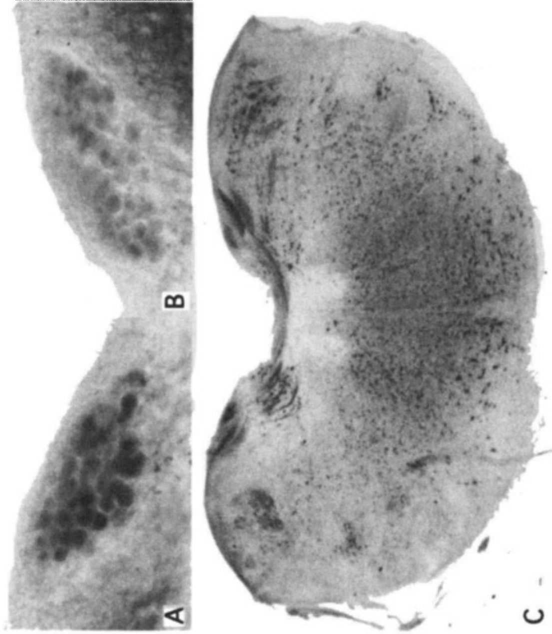


Figure 11. A normal developmental increase in expression of calretinin (CR) mRNA in the brainstem auditory nuclei is illustrated by darkfield photomicrographs of *in situ* hybridization autoradiographs on coronal sections through the chick brainstem at embryonic days (E)9 (**A**), 11 (**C**) and 17(**E**); calibration bars = 0.5 mm. Panels B, D and F show brightfield photomicrographs of CR-immunoreactivity at, respectively, E9, E11, and E17; calibration bars = 0.1 mm. NA, nucleus angularis; NL, nucleus laminaris; NM, nucleus magnocellularis; Ta, nucleus tangentialis; VeD, descending vestibular nucleus; VeM, medial vestibular nucleus. The bottom part of the figure is a dot blot comprising serial dilutions of NM homogenates from embryos of three ages (along with purified CR standards) stained with an anti-CR antibody. The dot blot confirms the findings of the other two methods that CR expression increases dramatically after the onset of synaptic function in NM at E11.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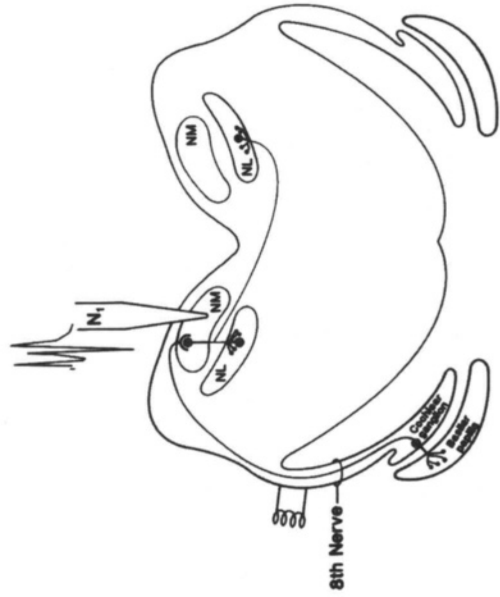
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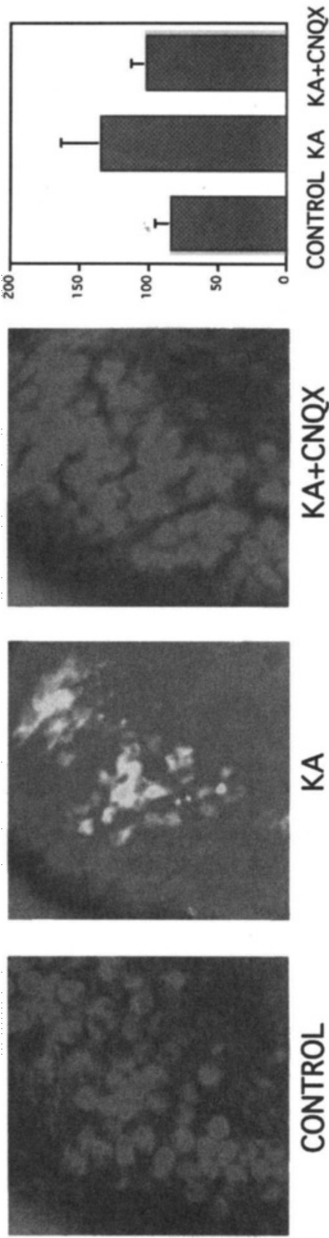
8th Nerve
NM
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are strongly influenced by the ear. Given the extensive experimental investigation of activity-dependent mechanisms in brain development over the last decade, it might be expected that general models would have been developed that could predict which features of a neural system are activity-dependent and which are not. It now appears, however, that there is such diversity within the CNS that each system of interest must be separately investigated to learn which features require activity and, when activity is important, whether it is permissive or instructive (Crair, 1999).

Electrical activity, of course, is only the most obvious thing that afferent auditory axons bring to their target neurons; there are undoubtedly non-neurotransmitter molecules released directly or indirectly by nerve activity (e.g., neurotrophins) that could affect target neuron development. This is a subject that has received very little study in the auditory system and deserves more attention from investigators. Most studies to date have been concerned with the signaling performed by neurotransmitter molecules acting on a variety of receptors coupled to a variety of intracellular effector mechanisms. As the exquisitely sensitive regulation of gene expression by patterned electrical activity has been revealed (Buonanno and Fields, 1999), it has become clear that the complex mix of spontaneous and stimulus-evoked activity to which auditory neurons are subjected provides a rich source of information for control of their development. For example, the glutamate signal released at most synapses in the ascending auditory pathway is transduced by a large number of functionally-distinct NMDA, AMPA and metabotropic receptors that can activate a variety of second-messenger systems to influence cell physiology and gene expression. Elucidating how the response of these receptors to the changing signals coming from the ear influences gene expression in developing auditory neurons is an important next step in understanding the ear's influence on the developing brain.

The information gained in such studies can also be expected to enlarge our understanding of the pathophysiologic mechanisms underlying neuronal loss in congenital deafness and identify new strategies for prevention and treatment.

7. ACKNOWLEDGMENTS

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Figure 12. Increased calretinin (CR) expression in nucleus magnocellularis (NM) following activation of synaptic AMPA receptors. **Top** panels show images of CR-immunoreactivity detected in NM by a calcium-insensitive primary antibody and TRITC-labeled secondary antibodies after 20 min *in vitro* exposure of brain slices to vehicle only (control), kainic acid (KA; 100 μM), or KA + 200 M CNQX (an AMPA receptor antagonist). The panel at the right shows the mean ± SD fluoresence measured from several adjacent sections in each animal. KA increases CR expression by 66% over control levels and the addition of CNQX substantially reduces the effect of KA. **Bottom left**, schematic diagram showing a coronal section through the chick brainstem auditory nuclei and (on the left side of this diagram) the in vitro brainstem slice preparation used for experiments in which the effects of synaptic stimulation on CR expression in NM were studied. A stimulating electrode was placed on one cochlear (8th) nerve and the efficacy of synaptic stimulation of the ipsilateral NM was monitored by recording the N_1 postsynaptic field potential; the contralateral NM is unstimulated under these conditions. **Bottom right**, high-power photomicrographs of CR-immunoreactivity on the stimulated (**A**) and unstimulated (**B**) sides of a single section (shown at low power in **C**) from an E15 brainstem that was unilaterally stimulated at 1.0 Hz for 1 hour, fixed in Bouin's fluid, and incubated with a calcium-insensitive primary antibody. The stimulated NM clearly shows stronger CR staining. Observers, who were unaware of the experimental treatments, scored the CR staining intensity on the two sides of 9 stimulated and 12 unstimulated sectioned brainstems and found statistically-reliable increased CR-immunoreactivity on the stimulated side of experimental animals (p =.012, Fisher's exact test).

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II

YOUTHFUL EXPOSURE

When the words "early experience" are uttered, one typically thinks about the sensory environment of the infant organism. The experimental evidence for a role of early sensory experience in development primarily comes from studies which evaluate the effects of early sensory deprivation on the development of a particular sensory system. The chapters contained in this section exemplify the approach of depriving developing neurons of the afferent activity that is driven by sensory nerves. This section break is, in essence, a fuzzy border, since the chapters intertwine extensively with those in the first section of this book. Themes such as cell death and the role of afferent activity in development will be revisited. In fact, from the perspective of mechanism, placing chapters in sections based on the time of the experimental manipulations is a somewhat artificial distinction.

In the first chapter of this section, Johnson and Leon (University of California, Irvine) describe the effects of early sensory experience on the development of the olfactory bulb. In some sense, this type of deprivation experiment is a prototypical example of what comes to mind when one thinks of manipulating the early sensory experience of an organism. However, the studies reported by Johnson and Leon are unusual and particularly impressive because the effects they observe are produced by a relatively minor manipulation of sensory experience. By simply plugging one naris, cells in the olfactory system will die. Equally impressive is the fact that enhanced exposure to odorants appears to increase the number of cells responsive to that odor. While it is still not understood exactly how this early olfactory experience controls the life and death of neurons, it is likely that it will involve activity-dependent alterations in gene expression. Johnson and Leon show how gene expression is altered by manipulations of sensory experience and present intriguing results on changes in the expression of one particular gene which is known to promote cell survival.

The chapter by Hyson (Florida State University) is similar to the Johnson and Leon chapter in that it describes how sensory deprivation early in life can lead to the death of neurons. The juxtaposition of these two chapters shows how different model systems (rodent olfactory system and chick auditory system) have both been used fruitfully to

begin to understand the cellular mechanisms underlying the effects of early sensory deprivation. The Hyson chapter also complements the previous chapter by Parks, in which it was shown that early deafferentation produces a variety of dramatic changes in the auditory system. Hyson focuses on one particular change resulting from deprivation and describes his attempts to identify the transneuronal signals responsible for that effect. He takes advantage of the fact that changes in the protein synthetic machinery of neurons in the chick's auditory system occur within hours after elimination of afferent activity. This rapid time course makes it possible to begin to understand the signaling mechanisms between pre- and postsynaptic cells that account for the afferent regulation of cell death.

The auditory system is also used in the studies described by Sanes, Kotek and Fitzgerald. Here again is the concern over how afferents control the development of the nervous system. However, Sanes, Kotek and Fitzgerald look at the development of inhibitory connections in the brain. This is an enormously important and frequently neglected issue. When the term 'activity-dependent' is used in the context of development one normally thinks of excitatory drive. Sanes, Kotek and Fitzgerald show that inhibition, too, has activity-dependent influences on development. The brain stem auditory system of the gerbil provides an elegant system for examining the development of inhibitory pathways. In this system, both excitatory and inhibitory projections converge onto neurons in the lateral superior olive, but they arrive there from anatomically distinct pathways and are driven by opposite ears. This allows discrete manipulations of the primary inhibitory projections to the cells. Their studies not only show that spontaneous inhibitory activity influences the development of this system, but that unusual transduction pathways may be used to exert this control.

3

EARLY INFLUENCES ON GUSTATORY DEVELOPMENT

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1. INTRODUCTION

The morphological and functional development of the rat gustatory system occurs primarily postnatally. As such, stimulus-induced processes likely play a major role in organizing the sense of taste. However, some very important events that direct development occur long before the gustatory system becomes functional. In order to examine some of the very early organizational processes, it has been necessary to institute experimental manipulations prenatally and then examine the effects as the system develops. Thus, by relating the neurobiological effects with prenatal manipulations, it is the hope that we can get a picture of events that serve to shape the long-term organization of the system during normal development. Moreover, as with all other sensory systems, it is important to identify processes that may have long-term (or permanent) detrimental effects on sensory and brain development. It is my goal in this chapter to provide background information about normal gustatory development that provides the necessary standard by which experimentally induced alterations can be compared, and to then describe findings resulting from early environmental manipulations.

2. NORMAL DEVELOPMENT OF THE GUSTATORY SYSTEM

2.1. Peripheral Functional Development

Neurophysiological taste responses change in a progressive and orderly manner throughout a prolonged period of the rat's postnatal development. In the peripheral gustatory system, chorda tympani nerve taste responses can be recorded as early as two days postnatal (Hill and Almli, 1980). However, response magnitudes to monochloride salt increase dramatically during the first three weeks after birth (Ferrell, Mistretta, and Bradley, 1981; Hill and Almli, 1980; Yamada, 1980). Specifically, response frequencies of

The Biology of Early Influences, edited by Hyson and Johnson
Kluwer Academic / Plenum Publishers, New York, 1999.

single chorda tympani fibers to NaCl and LiCl increase while frequencies to NH_4Cl remain constant (Hill, Mistretta, and Bradley, 1982). Interestingly, response frequencies to citric acid decrease throughout development. Thus, the peripheral gustatory system is capable of responding in a mature manner to some stimuli (e.g., NH_4Cl) as soon it becomes functional, but changes in its sensitivity to others (e.g., NaCl) during an extended developmental period (Fig. 1).

The cellular mechanisms responsible for the increase in peripheral response frequencies to NaCl relate to the addition of functional taste receptor membrane components that are sensitive to the epithelial sodium transport blocker, amiloride (Hill and Bour, 1985) (Fig. 1). This sodium transporting channel has been characterized in other epithelia (Garty and Benos, 1988), and has been implicated as the major, if not only membrane component mediating salt taste transduction in the rat (Avenet and Lindemann, 1988; DeSimone and Ferrell, 1985; Formaker and Hill, 1988; Hill, Formaker, and White, 1990; Ye, Heck, and DeSimone, 1991). Recent experiments that combine *in vivo* voltage clamp with whole nerve electrophysiological recordings from the chorda tympani nerve have provided further evidence of increased sodium transduction pathways during development. Briefly, the voltage applied across the lingual epithelia is clamped at positive or negative voltage, thereby decreasing or increasing the driving force, respectively, for sodium to enter the taste receptor cell. By simultaneously recording from the nerve, it is possible to examine the consequences of the voltage clamp during salt stimulation and to derive biophysical measures of receptor channel density and efficiency (Stewart, Hendricks, Heck, DeSimone, and Hill, 1997). These experiments reveal that there are dramatic increases in the numbers of functional amiloride sensitive sodium channels that are located in the apical portion of taste receptor cells (i.e., the area most likely to contact gustatory stimuli). Specifically, the combined neurophysiological and biophysical analyses show that there are approximately 19%, 44%, and 85% of functional amiloride channels in rats aged 10–14 days, 19–23 days, and 29–31 days, respectively, compared to adults.

Interestingly, immunocytochemical and whole cell patch clamp evidence suggests that at least part of the amiloride-sensitive channel is present in rat fungiform taste buds at birth (Stewart, Lasiter, Benos, and Hill, 1997). Immuno-positive labeling with a polyclonal antibody directed at the epithelial sodium channel (ENaC) is evident in taste buds beginning in 1-day-old rats and are qualitatively unchanged throughout development.

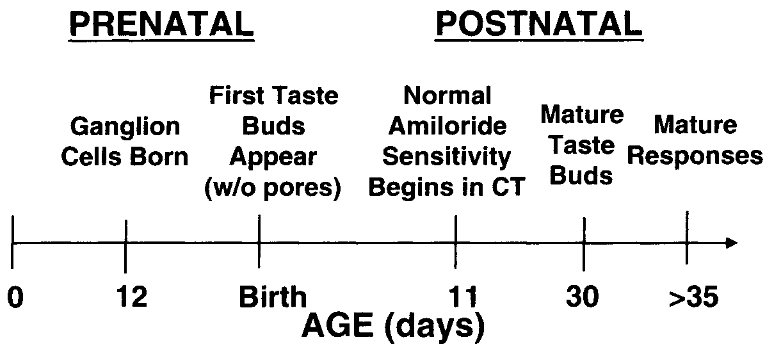

Figure 1. Time scale showing important events in the functional and morphological development of the peripheral gustatory system. CT—chorda tympani nerve.

Thus, it appears that at least some components (i.e., subunits) of the receptor are present very early in development. In fact, there is evidence that these channels may be functional but not located appropriately in taste cell membranes. Recent *in vitro* work using patch clamp recording techniques of isolated taste buds demonstrate that amiloride-blockable currents are present in taste receptor cells of newborn rats, and that the same proportion of taste bud cells with functional amiloride sensitive channels are the same as in adults (Kossel, McPheeters, Lin, and Kinnamon, 1998). The authors of the patch clamp study suggest that both the immunocytochemical results and the electrophysiological results can be reconciled with the apparent discordant neurophysiological findings in that functional amiloride channels in immature rats do not have sufficient access to stimuli. Specifically, functional channels located other than in the apical portion of receptor cells would not be stimulated by sodium and amiloride. Regardless of the actual mechanism, it is apparent that a developmental process(es), perhaps hormonally mediated (Stewart and Hill, 1993), must occur during the first two weeks postnatal to make the channel functional in the apical domain of taste buds. It is the dramatic increase in sodium and concomitant amiloride sensitivity with age that represents the major functional hallmark of changes in the anterior tongue (Fig. 1).

2.2. Central Functional Development

As observed in the peripheral gustatory system, central nervous system responses from the rat nucleus of the solitary tract (NST) in the medulla, and parabrachial nuclei (PBN) in the pons can be recorded within the first postnatal week (Hill, 1987a; Hill, Bradley, and Mistretta, 1983). Moreover, major changes occur in CNS taste responses throughout an extended period in the rat's preadult life (Hill, 1987a). However, response maturation primarily to sodium salts occurs at a later age in NST and PBN neurons than in chorda tympani neurons. While mature responses to NaCl occur in chorda tympani neurons before 24 days postnatal, mature responses do not occur in NST and PBN neurons until after 25–35 days postnatal. Therefore, changes in NST and PBN responses reflect peripheral changes as well as distinct alterations in the central nervous system. These functional studies collectively show that the developing gustatory system changes the most to sodium salts during the first three postnatal weeks.

2.3. Peripheral Morphological Development

Along with peripheral and central neurophysiological taste response maturation, concomitant developmental changes in morphology occur postnatally in the rat (Fig. 1). Gustatory papillae and associated taste buds initially form in the rat fetus before or near term, but taste buds do not fully mature structurally until approximately thirty days postnatal (Farbman, 1965; Farbman and Mbiene, 1991; Gottfried, Mistretta, Bradley, and Hill, 1984; Mbiene and Farbman, 1993; Mistretta, 1972). Therefore, some aspects of taste receptor maturation may be complete during the second through the fourth week after birth; yet, adult-like functional responses are not obtained until later in development (Hill, 1987a).

Adult-like innervation patterns of fungiform taste buds are also not evident until after 40 days postnatal. For fungiform papillae located on the anterior tongue in adult rats, there is a very high correlation between the number of chorda tympani neurons that innervate taste buds and the size of the taste bud—the larger the taste bud, the more

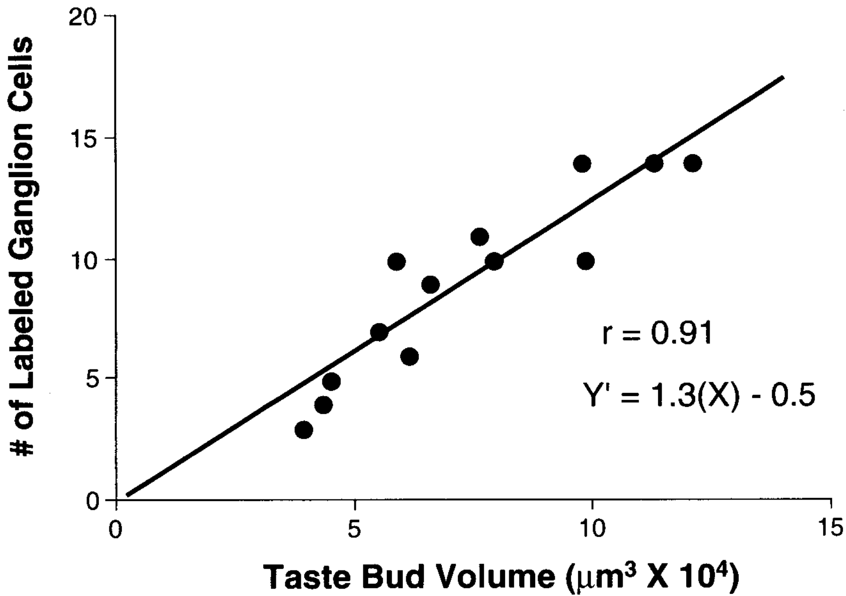

Figure 2. The number of labeled geniculate ganglion cells are plotted against taste bud volume for taste buds sampled from the mid-region of the adult tongue. The regression line is shown as a solid line and the correlation coefficient and regression equation are shown in the lower right portion of the figure. (From Krimm and Hill, 1998)

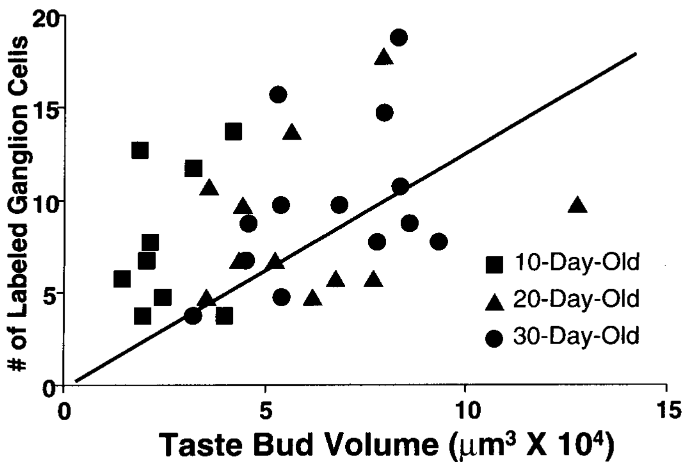

Figure 3. The number of labeled geniculate ganglion cells plotted against taste bud volumes sampled from the tongue mid-region in 10-day-old (squares), 20-day-old (triangles), and 30-day-old rats (circles). The regression line is replotted from Figure 2. There is no relationship between taste bud volume and number of labeled ganglion cells at any of these postnatal ages. (From Krimm and Hill, 1998)

chorda tympani neurons that innervate it (Krimm and Hill, 1998a) (Fig. 2). Such a function is not present in rats aged 10, 20, or 30 days postnatal (Fig. 3). In these three age groups, papillae are smaller than in adults and are innervated by a disproportionately large number of chorda tympani fibers compared to adults. Therefore, the number of neurons "match" the size of the taste bud only after 40 days postnatal. While the relationship between number of innervating neurons and taste bud size is not achieved until relatively late in postnatal development, it appears that neurons innervating a single taste bud before 10 days maintain their contact with that taste bud throughout the life of the animal (Krimm and Hill, 1998b). That is, significant neural arrangements do not occur after 10 days postnatal. So, what is the emergence of the relationship between taste bud size and number of innervating neurons due to? The answer may be simple—taste buds grow through 40 days postnatal to match the number of neurons that innervate it (Fig. 4). This brings up the interesting possibility that the mature numbers of innervating neurons are established during prenatal and/or early postnatal development by factors resident in developing gustatory tissue (e.g., neurotrophic factors). The differential amounts of releasable factor from the early taste bud may then dictate the mature complement of chorda tympani fibers. Finally, factors released from the available neurons may then determine the ultimate size of the taste bud and form the relationship between number of innervating neurons and taste bud size. This obviously would require significant interactions between peripheral gustatory fibers and their targets. Such a match between taste buds and individual neurons also seems present in receptive field development (i.e., the number of taste buds a single chorda tympani fiber innervates), as identified by studies of the sheep gustatory system (Nagai, Mistretta, and Bradley, 1988). There is a reorganization of chorda tympani receptive fields during sheep development from 130 days gestation through about 2 months postnatal. During this period, receptive fields change from a large, diffuse pattern to a smaller, more specific pattern. These developmental changes occur concomitantly with an increase in the proportion of chorda tympani fibers that respond maximally to NaCl compared to those responding maximally to NH_4Cl or to KCl. Moreover, neurons with the smaller receptive fields have higher response frequencies to NaCl than those with larger receptive fields.

2.4. Central Morphological and Behavioral Development

Along with these developmental changes in peripheral innervation patterns, morphological characteristics of the central terminal field organization also change with age. The terminal field of the chorda tympani nerve in the NST is evident at birth, yet it expands in a caudal direction throughout the first 3 weeks postnatal. Similarly, the dendritic expansion of neurons in the NST and in the next central gustatory relay, the PBN, is not complete until 20 and 45 days postnatal, respectively (Lasiter and Kachele, 1988; Lasiter, Wong, and Kachele, 1990). As such, it is clear that the developmental program for each neural structure is unique and may obey a serial organizational scheme.

The neurophysiological and morphological alterations that occur during development suggest that behavioral responses to some taste stimuli, especially to NaCl, also change postnatally. Indeed, rats are capable of responding behaviorally to some stimuli soon after birth. However, quantitative (i.e., intensity) and qualitative (e.g., saltiness) stimulus characteristics change dramatically during a prolonged period of development (e.g., Bernstein and Courtney, 1987; Formaker and Hill, 1990; Midkiff and Bernstein, 1983; Moe, 1986). Moreover, the behavioral changes to sodium appear to be mediated

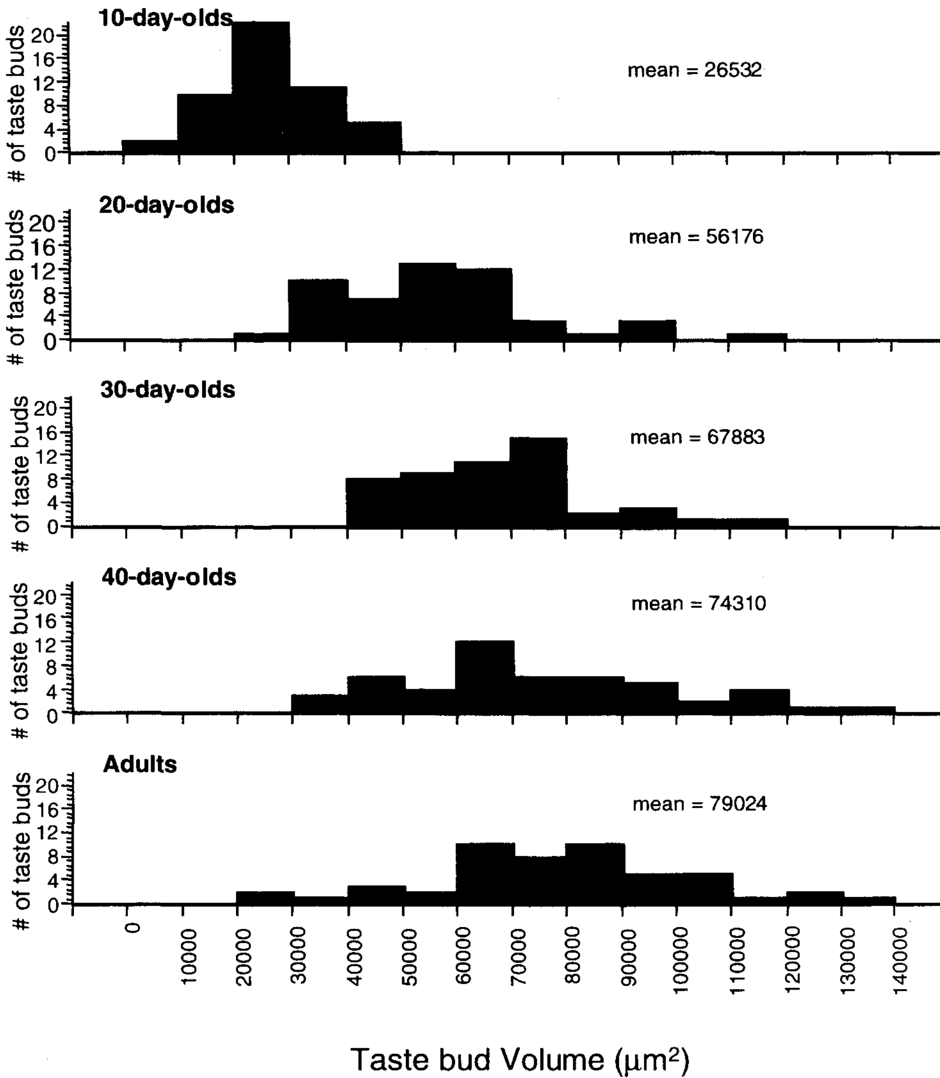

Figure 4. Frequency distribution of taste bud sizes during development. Taste buds increase in mean volume and in the range of volumes as age increases up to 40 days postnatal. (From Krimm and Hill, 1998)

by the functional development of the amiloride-sensitive sodium channel, as predicted from electrophysiological findings (Hill *et al.*, 1990).

In summary, the normal functional, morphological and behavioral development of the rat gustatory system occurs almost exclusively postnatally. The most dramatic functional and behavioral changes occur in response to sodium salts. Importantly, we know more about the neurobiological mechanisms involved in sodium taste development than for any other stimulus. Due to these properties, sodium salts appear to be ideal stimuli that can be manipulated during development in order to gain insights into some of the organizational properties needed to establish the neurobiology of taste.

3. ENVIRONMENTAL INFLUENCES ON THE DEVELOPING GUSTATORY SYSTEM

3.1. Early Dietary Influences on Peripheral Functional Development

From research of other sensory systems, we know that normal functional and morphological sensory maturation depends upon proper stimulation during well-defined periods of development. During these "critical" developmental periods, the neural apparatus can be modified easily (Aslin, 1981; Mistretta and Bradley, 1978). An extensive literature exists which examines the consequences of sensory restriction during development and the reversal of such effects in the olfactory, auditory, visual, and somatosensory systems (e.g., Brunjes and Frazier, 1986; Deitch and Rubel, 1984; Hubel and Weisel, 1970; Renehan, Rhoades, and Jacquin, 1989). These have been important studies not only in elucidating the capacity of the respective sensory system to respond to abnormal environmental conditions, but also to understand the processes necessary for normal development. That is, the goal of much of this work is to learn about normal development by perturbing the system. In comparison to other sensory systems, little emphasis has been placed on clarifying the role of sensory experience in the developing gustatory system. A major focus of work in our laboratory has been to provide the framework for understanding how environmental events relate to gustatory development. As detailed below, we have focused on environmental effects on sodium taste.

Restriction of maternal dietary sodium, beginning on day 3 postconception and continued in offspring throughout development results in dramatically reduced neurophysiological responses to sodium salts in the chorda tympani. Responses to NaCl are reduced by as much as 60% in sodium-restricted rats compared to control rats. In contrast, taste responses to NH_4Cl and nonsalt stimuli are unaffected. Thus, it is as if the developing gustatory system's response to sodium salts are functionally "frozen" at an immature age. The reason for the selective decrease in sodium salt-elicited responses is the absence of functional amiloride-sensitive sodium channels (Hill, 1987b; Hill, Mistretta, and Bradley, 1986; Hill and Przekop, 1988). We have provided corroborative data by combined *in vivo* voltage clamp and whole nerve neurophysiology on the mechanism responsible for the lack of sodium sensitivity by showing that the percentage of channels in the apical domain of taste receptor cells in restricted rats is approximately 10% of those in controls (Ye, Stewart, Heck, Hill, and DeSimone, 1993). Thus, a functional taste receptor cell transduction component can be modified by early dietary manipulations. This receptor modification, in turn, results in an altered afferent message transmitted to the central gustatory system. Importantly, the period of environmental vulnerability occurs long before the initial appearance of taste buds on the anterior tongue (Hill and Przekop, 1988). Specifically, if the diet is instituted on day 3 or day 8 postconception and the mothers and pups are maintained on the diet throughout development, the reduced chorda tympani response to sodium salts is produced. A delay in 2 days or longer to institute the diet, however, results in a normal developing chorda tympani response (Figs. 5 and 6). Therefore, a "sensitive" period exists soon after conception that has a dramatic influence of taste receptor function postnatally. This period is especially instructive, given the normal time course of peripheral gustatory development. The end of the "sensitive" period (i.e., 8 days postconception) is before the cell soma of the chorda tympani nerve located in the geniculate ganglia are born (approximately E12) (Altman and Bayer, 1982) and long before taste buds appear on the tongue and become functional. Therefore, this early embryonic "sensitive" period strongly suggests that stimulus-receptor interactions

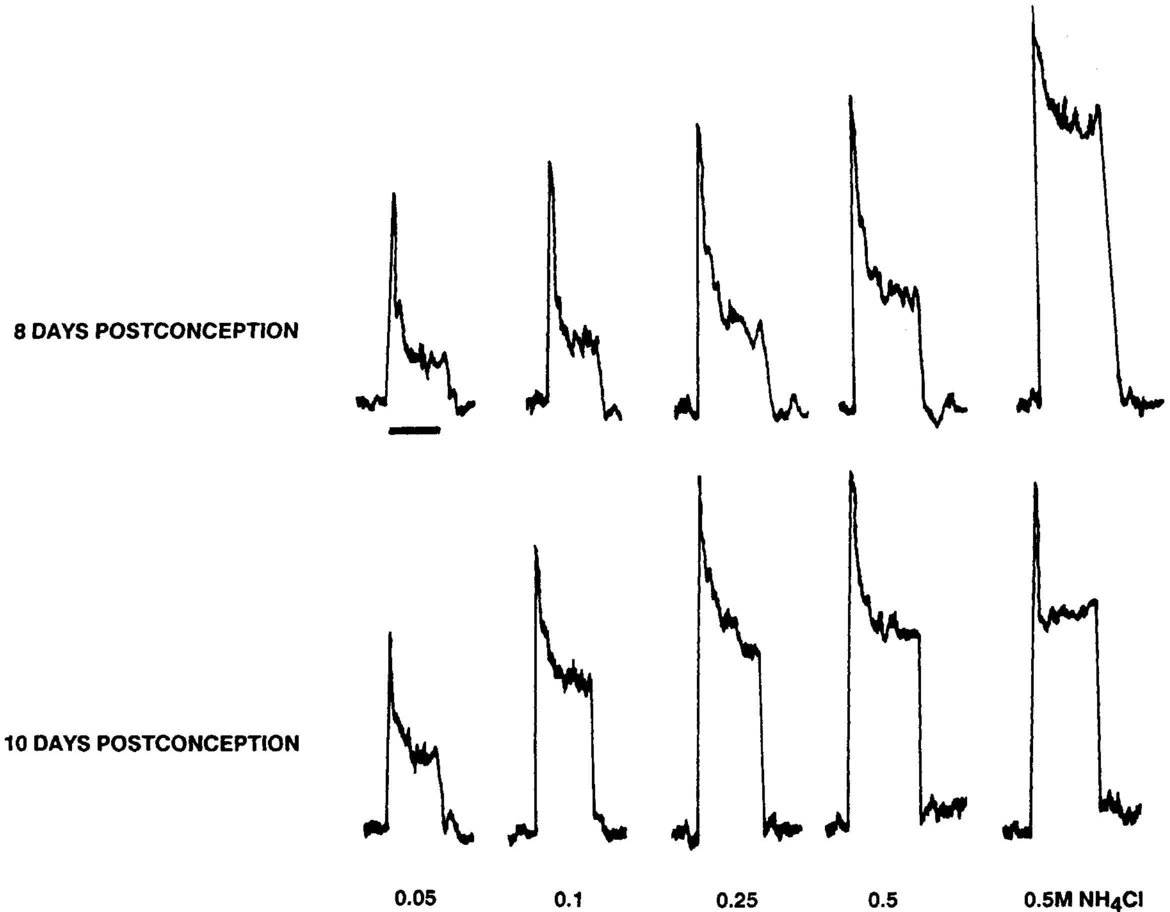

Figure 5. Integrated responses from the chorda tympani nerve to a concentration series (0.05 M to 0.5 M) of NaCl and 0.5 M NH_4Cl in a rat that the 0.03% NaCl diet was imposed at 8 days postconception (top) or at 10 days postconception (bottom). Scale bar is 15 s. (From Przekop and Hill, 1988)

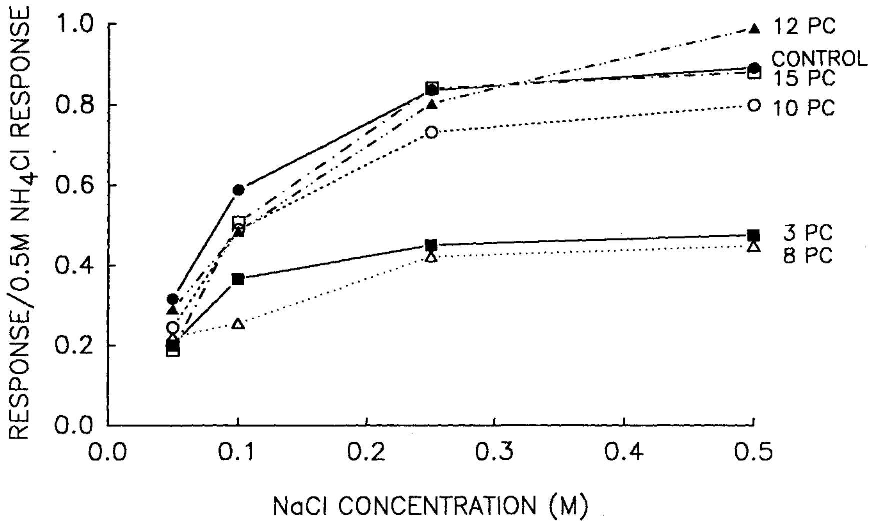

Figure 6. Mean response ratios from the chorda tympani nerve to a concentration series of NaCl in rats sodium restricted at postconception day 3, day 8, day 10, and day 15, and from rats fed the control diet throughout development. (From Przekop and Hill, 1988)

are not crucial for the formation of amiloride-sensitive sodium channels. Instead, these results indicate that circulating agents such as hormones and growth factors play an important role in determining the response properties of developing taste receptor cells. Moreover, the early dietary manipulation instituted within the first week postconception may have an effect on a physiological system(s) that ultimately impact on the developing taste system when amiloride sensitivity begins, at approximately 11 days postnatal (Fig. 7). Indeed, immunocytochemical evidence suggests that some components of the channel are present in the taste receptor cells of both sodium-restricted and normal rats (Stewart *et al.*, 1995). Thus, like normal newborn rats, taste receptor cells are immunocytochemically positive for the amiloride-sensitive channel antibody but are not functional. It may be that circulating factors trigger the functional development of these channels in normal rats, but are precluded from doing so in sodium-restricted rats. To date, however, the list of candidate factors that would be affected by early sodium restriction that also regulate amiloride-sensitive sodium channels is long and have not been examined for their role in regulating transduction elements the developing salt taste system.

Although these initial effects on taste receptor cell function are dramatic, the dietary effects on the peripheral gustatory system are reversible. Chorda tympani responses in restricted rats fed a NaCl-replete diet recover to control levels within 15 days, with a corresponding increase in functional amiloride-sensitive sodium channels (Hill, 1987b). Restoration of function during adulthood can also be induced by a single ingestive bout of physiological saline and by injected anesthetics (Przekop, Mook, and Hill, 1990; Stewart and Hill, 1990; 1996). In the case of saline ingestion, recovery is not dependent on direct stimulation of taste receptor cells with sodium, but relates to consequences of sodium intake independent of taste stimulation. Rats allowed to ingest an amount of sodium sufficient to restore chorda tympani function but not allowed to absorb it, fail to recover chorda tympani function (Przekop *et al.*, 1990). Obviously, the anesthetic-induced recovery does not require receptor contact with sodium either (Stewart and Hill, 1990).

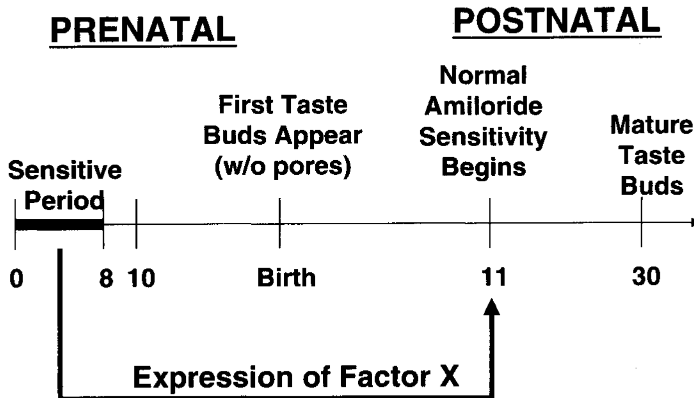

Figure 7. Time scale showing important events in the functional and morphological development of the peripheral gustatory system, including the early prenatal "sensitive period". This early period suggests that factor(s) (e.g., growth factors) influenced soon after conception have their actions on the taste system later in development.

Therefore, these results further support the hypothesis that the sodium transducer is under hormonal or growth factor control and that ingestion and absorption of sodium is necessary for the development of sodium taste.

3.2. Early Dietary Influences on Central Morphological Development

In as much as the NaCl-deficient diet has such profound effects on the functional development of the periphery, it appears that such environmental alterations have even more prominent effects on central gustatory system development. Dietary sodium restriction during pre- and postnatal development produces both abnormally distributed and irregularly shaped chorda tympani terminal fields (King and Hill, 1991). In rats sodium restricted from 3 days postconception throughout development, the terminal field of the chorda tympani nerve in the rostral pole of the NST is irregularly shaped and is disproportionately large compared to controls. Once restricted rats are placed on a sodium replete diet at approximately 28 days postnatal, the terminal field increases even more than in restricted rats. In fact, the terminal field volume of "recovered" rats exceeds that of controls by three times (Fig. 8).

We have recently determined that the dietary effects on terminal field development are not evident until after weaning (approximately 25 days postnatal). Therefore, restricted rats have a normal chorda tympani field soon after birth and only show the abnormal field much later, suggesting that the later emerging effects are activity related (unpublished findings) (Fig. 8). Lack of proper stimulus-elicited responses (i.e., sodium salt responses) may be necessary to produce a normal field. We have also examined the morphology of single chorda tympani fibers as they project to the anterior NST in control, restricted and "recovered" rats. Briefly, all groups have central fibers that are relatively simple in morphology and do not branch extensively. Moreover, it does not appear that there are differences between the groups in their morphology. Rather, chorda tympani fibers in restricted and "recovered" rats project further caudally in the NST compared to controls (unpublished findings). Therefore, it is not the morphologies of the individual fibers that are affected by the early dietary manipulations; rather it is where

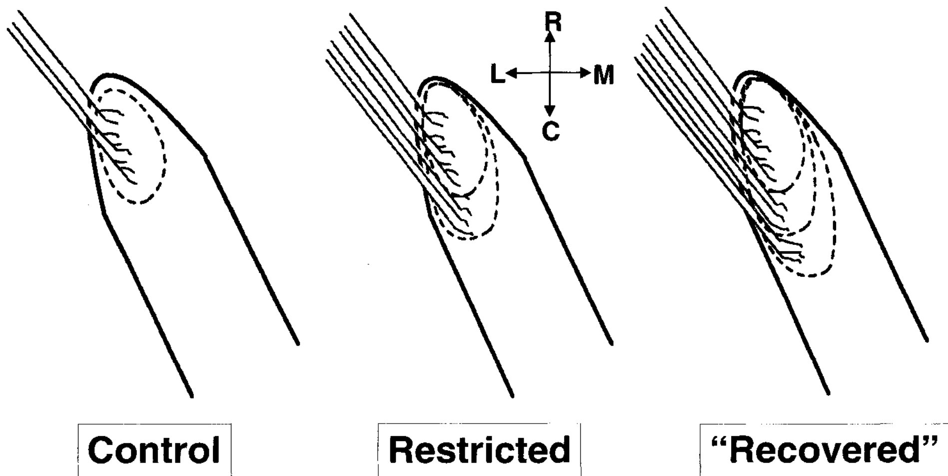

Figure 8. Schematic of the chorda tympani (CT) field in the rostral pole of the nucleus of the solitary tract (NST). The left hemifield of the NST in horizontal section is shown by the solid lines for control, restricted and "recovered" rats. The dotted lines in each NST refer to successive expansion of the chorda tympani terminal field from controls to "restricted" rats. The proposed organization of chorda tympani fibers are shown as they project into the NST. Restricted rats have an enlarged terminal field compared to controls and have chorda tympani fiber morphologies similar to controls, but extending more caudally. Similarly, the terminal field in "recovered" rats extends more caudally than in restricted rats, with control-like fibers terminating more caudally than in restricted and control rats. R—rostral; C—caudal; M—medial; L—lateral.

the neurons make their projections into the NST. Chorda tympani fibers in restricted and "recovered" rats simply project more caudally than those in control rats.

From our functional studies, we know that an early "sensitive" period exists whereby failure to place rats on a sodium restricted diet before postconception day 8 will fail to produce lowered sodium responses in the offspring. We have also identified a very early critical period in chorda tympani terminal field development. However, unlike experiments that examined the functional sensitive period where restricted rats were not returned to a sodium replete diet, these rats were fed the sodium restricted diet beginning at 3 days postconception and continued until it was replaced with the sodium replete diet at E9, E12, E15, birth or 28 days postnatal. What we found was very unexpected. A brief, early prenatal period of sodium restriction from postconception day 3 to 12 is sufficient to produce a permanent alteration in the chorda tympani terminal field (Krimm and Hill, 1997). Longer periods of sodium restriction produced similar amounts of terminal field expansion. Thus, a permanent presynaptic morphological alteration at the first central gustatory relay occurs as a result of dietary manipulations initiated during a limited time when chorda tympani neurons are born (Altman and Bayer, 1982). It is critical to note that the central anatomical effects are limited to the chorda tympani field; the size and topography of the projections from another gustatory nerve, the lingual-tonsilar branch of the glossopharyngeal, are unaffected by dietary manipulations (King and Hill, 1991).

In addition to the presynaptic changes in the NST, striking morphological and physiological alterations postsynaptic to the chorda tympani occur in developmentally restricted rats. Large multipolar and fusiform neurons in the rostral pole of the NST show pronounced increases in dendritic length and number (multipolar), while there is

no impact of sodium restriction on ovoid cells. The large multipolar and fusiform cells remain enlarged after rats are fed the NaCl-replete diet for 2–3 months; however, ovoid cells show remarkable increases in the number and length of their dendrites upon restoration of dietary sodium (King and Hill, 1993). Significantly, neurons affected by dietary restriction are believed to be relay neurons (Davis and Jang, 1986; 1988; Lasiter and Kachele, 1988; Whitehead, 1986). These data strongly suggest that the morphological effects of early dietary manipulations are specific to certain cell types and may relate to their function. Finally, we have recently found that the terminal fields of NST neurons in the next gustatory relay, the parabrachial nuclei (PBN) are similar among control, sodium restricted, and "recovered" rats (Walker and Hill, 1998). It appears, therefore, as though the PBN is resistant to the dietary-induced changes seen at the lower neural levels. We suggest that this resistance may be due to different developmental processes, and convergence of relatively more heterogeneous inputs in the PBN.

3.3. Early Dietary Influences on Central Functional Development

In concert with the morphological changes, the consequences of the early environmental manipulations are also expressed functionally. In restricted rats, NST neurons respond with lower response frequencies to sodium salts, while non-sodium salts and non-salt stimuli elicit normal responses (Vogt and Hill, 1993). Furthermore, there appears to be a shift in the stimulus to which NST neurons in restricted rats respond "best". Perhaps most significantly, however, is that restricted rats fed a NaCl-replete diet for at least 5 weeks at adulthood (i.e., "recovered" rats) have NST neurons that are hyper-responsive to sodium salts. Accordingly, these neurons have an apparent shift in the stimulus to which they respond best. Simply put, NST neurons shift their sensitivity to sodium salts from poorly responsive (in sodium-restricted rats) to hyper-responsive (in "recovered" rats). It must be emphasized that the central functional changes, like the morphological changes, appear to be permanent and relate to an alteration in only one component of the afferent taste message (i.e., sodium salt responses). Thus, it is not simply an overall decrease in stimulus-induced responses in restricted rats that have an effect on central taste development.

3.4. Stimulation-Specific Alterations in Central Morphology

While our strategy for experimentally manipulating the developing gustatory system has focused on prenatal dietary manipulations, important studies by others have also been done by manipulating the amount of taste experience in early postnatal rats. The focus of these studies has been to examine central morphological development. Specifically, Lasiter (1995) has examined the sensitive periods that occur postnatally for terminal field development in the NST and have determined the limits of stimulation that direct development. By using techniques whereby early postnatal pups are artificially reared from their mother, the amount and type of stimulation can be delivered in a precise way during specific periods of development. When water is used as the only taste stimulus provided to pups aged 4–10 days, the terminal fields of the combined chorda tympani and greater superficial petrosal nerves (i.e., facial nerve) in the NST are substantially smaller than in controls. This is in contrast to the enlarged fields produced by the early dietary manipulations described above. (This apparent discrepancy will be discussed later.) Stimulation with 30 mM, 150 mM or 300 mM NaCl or with lactose or whole rat milk results in normal sized facial nerve terminal fields in the rostral NST. Thus, there is

a strong indication that sufficient stimulation with specific stimuli during early periods of postnatal development are important in establishing the terminal fields of gustatory nerves that innervate the anterior oropharynx during early development. It is also very important to note that NaCl is a stimulus that will direct normal development of the terminal field. This is somewhat surprising given the relatively low afferent signal produced in the chorda tympani nerve during the period of stimulation (days 4–10 postnatal). Lasiter (1995) also determined the duration of stimulation by NaCl that influenced terminal field development of the facial nerve in the NST. Using 150 mM as the stimulus in artificially reared rats, he determined that 3 days of periodic stimulation is sufficient to produce normal terminal fields. One day of periodic stimulation failed to produce a normal field. Thus, there appears to be an important postnatal period in which taste stimulation is necessary to produce the terminal fields in the rostral NST.

3.5. The Two Sensitive Periods Involved in NST Terminal Field Development

Due to the opposite effects of early embryonic sodium restriction and postnatal artificial rearing on terminal field development (i.e., expanded and decreased fields, respectively), it appears as though the two experimental procedures are affecting different processes of central gustatory development (Fig. 9). It is likely that each of the two critical periods is important for separate, yet related, aspects of central gustatory development. The early embryonic critical period as revealed by dietary sodium restriction may dictate global processes of NST development and not the finer aspects of terminal field development directly. For example, the dietary manipulation may have its effect on developmental processes that occur very early in NST development, such as placement of the boundaries for the different incoming afferent nerves. Thus, by expansion of the molecular boundaries that cordon off one terminal field from others, the afferents that make their projections to the enlarged projection area would be able to expand beyond their normal boundaries. Stimulus-induced activity present during postnatal development may then influence how individual primary afferent fibers project to the area of the NST determined during embryonic development. Therefore, there must be a coordination of the pre- and postnatal influences that determine the final configuration and size of the terminal field. Processes during the prenatal critical period could determine more global aspects of NST development (i.e., prenatal dietary influences), while processes

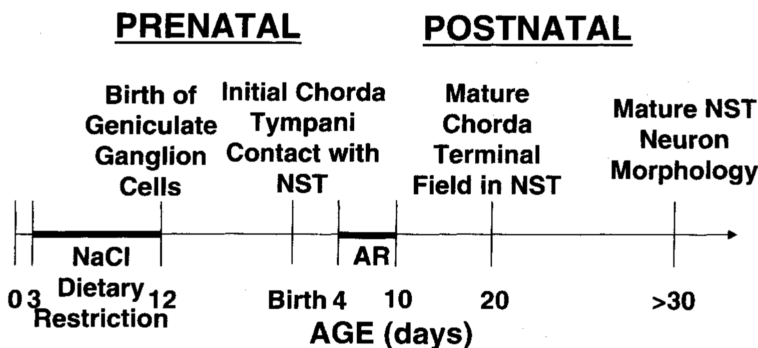

Figure 9. Time scale showing important events in the morphological development of the central gustatory system, including the early prenatal dietary-induced "sensitive period" (NaCl Dietary Restriction) and the early postnatal experience-dependent "sensitive period" (Artificial Rearing; AR)

during the postnatal critical period determines the finer aspects of terminal field organization (i.e., stimulus-induced processes).

3.6. Functional and Morphological Comparisons with the Greater Superficial Petrosal Nerve

As seen throughout this chapter, most of the functional and morphological studies on the developing gustatory system have focused on anterior tongue stimulation and/or processes related to the chorda tympani nerve. The primary findings from these collective studies have been the influence of sodium salts on the development of taste function and structure related to anterior tongue taste receptors. Through all of these studies, the development of functional and morphological characteristics related to other gustatory nerves has gone relatively unstudied. This may be due in part to the prevailing view that afferent information about salt (the stimulus that appears to change the most in its effectiveness during development) is carried by the chorda tympani nerve and not by other gustatory nerves. The other gustatory nerves are viewed to be primarily responsible in transmitting information about other taste qualities. For example, the greater superficial petrosal nerve (GSP), which innervates taste receptors on the palate and nasoincisor duct, has traditionally been considered the "sweet" nerve in that in conveys information about sugars and sweeteners to the brain relatively more effectively than other gustatory nerves. Similarly, the glossopharyngeal nerve conveys information primarily related to "bitter" tasting compounds. We have found recently, however, that the GSP also responds vigorously to salts and that the salt response is inhibited by amiloride. The relative response magnitude to NaCl compared to NH_4Cl in the GSP is similar to that in the chorda tympani nerve. Increases in NaCl stimulus concentration results in increases in GSP responses even beyond that for the chorda tympani. Moreover, the amount of response suppression by amiloride is similar to that in the chorda tympani (Sollars and Hill, 1998) (Fig. 10a). Thus, because the GSP and the chorda tympani nerve share many of the response properties to NaCl and because the terminal fields of the GSP and chorda tympani are in the rostral pole of the NST, the GSP would be expected to be as susceptible to environmental manipulations as would the chorda tympani. Interestingly, neither the function of the GSP or the terminal field are affected by dietary sodium restriction when the sodium restricted diet is instituted at 3 days postconception and continued throughout development (unpublished findings). The response-concentration functions for salt stimuli are similar between restricted and control rats (Fig. 10b). Likewise, the terminal field of the GSP is unaffected by the early dietary manipulation (unpublished findings). The latter finding may not be surprising if terminal field development is dependent upon proper activity in the primary afferent nerve. That is, normal function in the GSP should lead to normal terminal field development in the NST, whereas decreased activity in the chorda tympani should lead to alter terminal field development in the NST. More importantly, these results suggest that the prenatal events that influence chorda tympani development do not affect GSP development. One nerve is resistant to the dietary manipulation while the other is not.

4. SUMMARY AND CONCLUSIONS

The largest functional changes that occur during normal development of the rat gustatory system relates to increases in responsiveness to sodium salt stimuli.

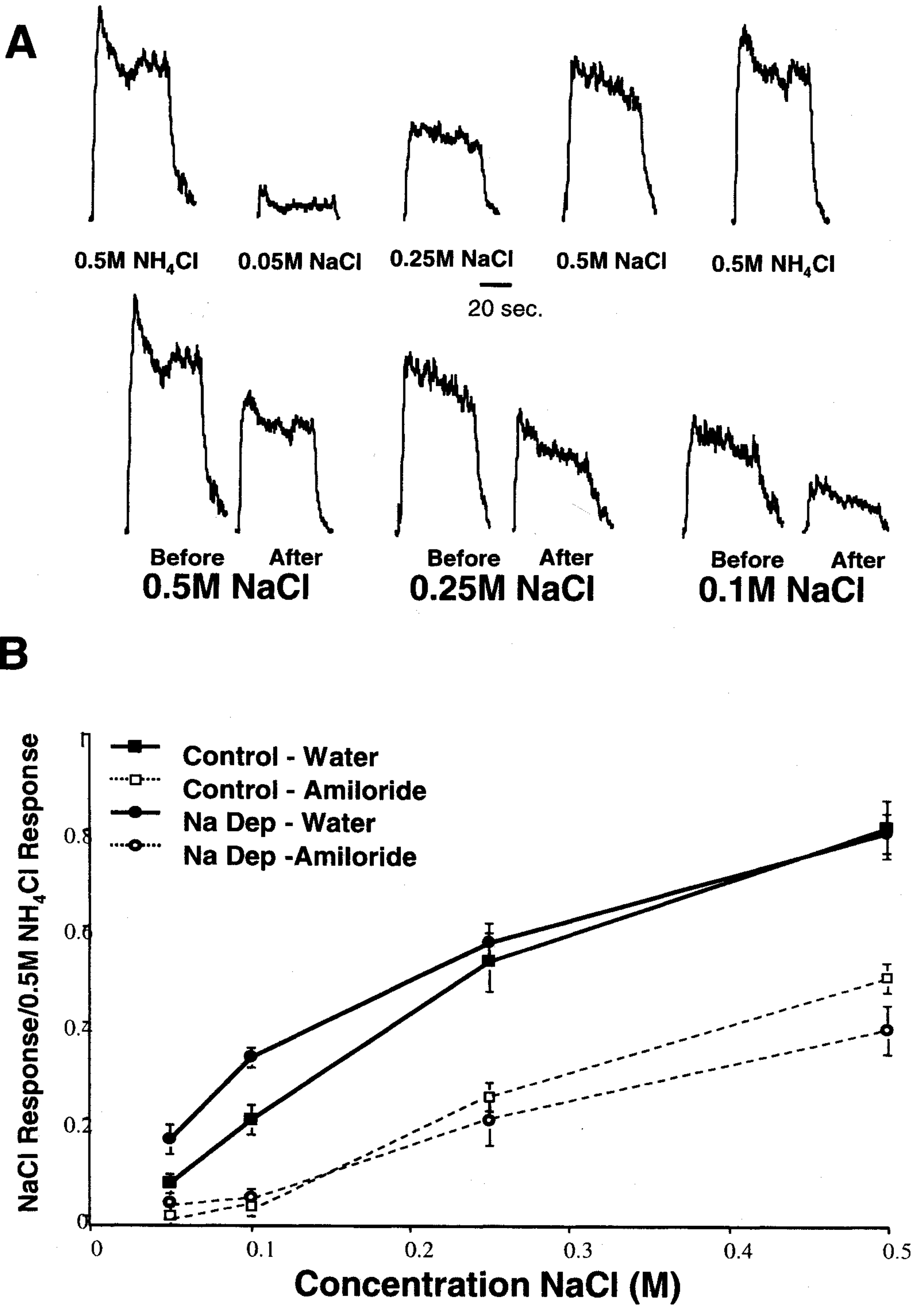

Figure 10. A. Integrated responses from the greater superficial petrosal nerve (GSP) in a control rat to a series of salts (top) and to three concentrations of NaCl before and after lingual application of amiloride (bottom). B. GSP response-concentration functions to NaCl in control and in developmentally sodium-restricted rats (Na Dep) before and after amiloride.

Manipulation of dietary sodium content during early prenatal development has profound influences on the functional and morphological development of the system. Namely, early sodium restriction "freezes" the functional development of the chorda tympani nerve in an immature state while allowing other responses to develop normally. The mechanism that appears to underlie the lack of functional development relates to the functional development of the amiloride-sensitive sodium channel. Importantly, the diet must be instituted during a very early period of embryonic development that may have influences on physiological systems other than the gustatory system, but impacts on the taste system postnatally. Morphologically, a very early, limited period of early dietary sodium restriction has a profound and permanent affect on the terminal field organization of the chorda tympani nerve in the NST. It is likely that this early period is when some of the organizational processes occur in the NST and are affected by the dietary manipulation. Another important critical period occurs early postnatally when activity seems to be required for terminal field development. Thus, at least two important periods are necessary for central morphological organization. Finally, these processes are somewhat specific for the chorda tympani. Another gustatory nerve, the GSP also is responsive responses to sodium salts and to amiloride, yet it is resistant to the early dietary effects of sodium. Comparing the developmental processes between the chorda tympani and the GSP may provide insights into the factors that are involved in the plasticity of the gustatory system.

5. ACKNOWLEDGMENTS

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III

TRANSFORMATIONS

It is widely appreciated that development is not merely the growth of an organism, but rather, it is characterized by a number of transformations in cell fate and function. In some cases, radical changes in neural structure and function can take place relatively "late" in the developmental scene. When dramatic transformations occur during a defined time period, it is easier to identify the signals responsible for the modification. The chapters in this section take advantage of model systems that undergo clear transformations in neural structure and function. One of the most extraordinary transformations observed in developing organisms is that of metamorphosis. Here, the organism completely redefines anatomical and behavioral phenotypes as is transforms, for example, from crawling larvae to fluttering moth. However, somewhat more subtle transformations also occur as juvenile organisms develop behavioral phenotypes such "sexually mature" or "socially dominant."

The first chapter in this section by Janis Weeks (University of Oregon) examines metamorphosis in the hawkmoth *Manduca sexta*. During the transition from caterpillar to moth, some motoneurons die, while others alter their anatomy, take on a new function in the pupa, and then die at a later time. The work on this system demonstrates that both extrinsic signals and features intrinsic to a given cell determine the developmental fate of the neuron. Unlike the results presented in many of the preceding chapters, it appears that extrinsic signals originating from cell-cell contact and communication are of little consequence. Instead, Weeks' work demonstrates the powerful role played by steroid hormones in this late developmental transformation. However, not all motoneurons respond in the same way to the hormonal fluctuations that drive metamorphosis. Some intrinsic feature, related to their larval segmental position, appears to determine whether the steroid will cause the motoneuron to initiate programmed cell death, or whether the cell will be transformed to take on a new function in the pupa.

Gail Burd (University of Arizona) presents another chapter which demonstrates the power of studying metamorphosis for understanding mechanisms of developmental change. This chapter examines metamorphic changes in the olfactory system of the African clawed frog, *Xenopus laevis*. The tadpole only requires an olfactory system

capable of detecting water-borne molecules, but after metamorphosis the frog must be able to detect both water-borne and air-borne odorants. The transformation of the olfactory system that meets this functional demand is simply phenomenal; an entirely new sensory epithelium is formed, while the old epithelium changes anatomically and molecularly, and even alters its central pattern of innervation. The studies of *Xenopus* metamorphosis reprises the idea that hormones (in this case thyroid hormone) are a common mechanism for late developmental transformations. However, in the case of the frog, it is also clear that cell-cell interactions remain a potent mechanism for guiding some of these later developing modifications.

Cell-cell interactions are also a prominent feature in the chapter by Frank Johnson (Florida State University). Here, the focus is the late changes in the morphology of a telencephalic region of zebra finches involved in song production. This area is initially monomorphic in males and females, but during juvenile life this region displays growth in males (which sing), and alternatively, substantial neuronal death in females (which do not sing). Since this transition in neural structure and behavior is both age-dependent and sex-dependent, it provides a powerful model for investigating the possible signals responsible for the life and death of neurons. When one thinks of sex differences, one typically thinks of the gonads. Work on the song bird, however, suggests that gonadal hormones are not the key feature for sex differences in brain structure and function. Rather, Johnson's work suggests that afferent input from another telencephalic region is responsible for the life and death of neurons during juvenile life and he has identified a trophic molecule which may play a role in promoting cell survival.

The emergence of new behavioral repertoires is a central theme in the final chapter by Donald Edwards (Georgia State University). His work addresses two of the most complex experiential influences examined in this book: learning and social interactions. Learned behavioral changes are probably the most obvious of all experience-dependent influences on an organism. Although most learned changes in behavior are not typically considered to be unique to developing organisms, understanding the factors leading to the emergence of learning capacities is an important developmental issue. In studies with crayfish, Edwards shows that a new behavioral potential, the ability to display habituation, gradually emerges in juvenile animals. Astonishingly, the biological change responsible for the emergence of this behavioral capacity is simply the growth of cells that comprise a neural circuit. Edwards also shows that experience-dependent changes in behavior and the nervous system can emerge from social interactions. When crayfish fight, their behavior and nervous systems are differentially modified depending on whether they win or lose. By ending with these studies we are forced to keep in mind that transformations of behavior and nervous system function do not end when we categorize an organism as "mature."

4

INFLUENCE OF EARLY SALT DIET ON TASTE AND BLOOD PRESSURE IN RATS

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1. INTRODUCTION

This chapter represents, in part, a progress report on a research program investigating the influence of prenatal and early postnatal exposure to NaCl on the development of adult taste preferences and aversions and blood pressure regulation in Sprague-Dawley rats. The research program's focus is on development and plasticity with the view that the structure and function of the neuroendocrine systems that control sodium and blood pressure regulation are immature during early stages of development and amenable to change due to dietary experience. The research program unites the effort of five independent investigators each of whom having unique expertise on sensory and regulatory behavior and neuroendocrine mechanisms. The investigators are Neil E. Rowland, Alan C. Spector, and Michael J. Katovich from the University of Florida, and James C. Smith and myself at the Florida State University. Robert M. Werner, a Research Veterinarian, directs an animal core facility at FSU for breeding and raising the experimental animals under strict dietary treatment conditions for the research of the five laboratories. The multiproject research program has been in existence for two years. However, the research has its historical roots in the literature dealing with the neuroendocrine mechanisms of motivation, particularly that dealing with hunger, thirst, and salt appetite.

1.1. Salt Preference and Salt Appetite

As a model system for the study of motivation, salt taste preference and need-driven salt appetite may be ideal for several reasons. The preference and appetite for salt appears to be relatively specific to sodium. Many organisms have a natural preference for the taste of salt that can be influenced by need and experience, and there is a sodium-sensing module within the gustatory system to identify sodium in the external environment. This salt taste-sensing module is an important component of an integrated and centrally orga-

The Biology of Early Influences, edited by Hyson and Johnson
Kluwer Academic / Plenum Publishers, New York, 1999.

nized neural system that mediates salt-seeking and salt-ingestion behaviors. Much of this research literature arises from homeostatic theory and its concepts, with behavior, and neural and hormonal systems being the arms of regulation orchestrated centrally primarily in lower forebrain and brainstem structures.

In the context of homeostasis, sodium is an essential nutrient required of all bodily processes, like neuronal function, cellular metabolism, and blood volume and blood pressure regulation to name a few critical examples. Inasmuch as sodium is the main constituent of the extracellular fluid, the body's internal sea, sodium and water regulation are inextricably linked. Through a complex system of input and output mechanisms, the body maintains optimal and proportional body sodium and water levels. Sodium and water must be ingested and/or excreted in the service of water and electrolyte balance or homeostasis. As a consequence, many mammals evolved with behavioral and neuroendocrine mechanisms to acquire and conserve sodium levels in the body.

With respect to sodium acquisition, several species, including humans and laboratory rats, evolved with a natural preference for the salty taste even in the absence of need. When given a two-bottle preference test between water and saline, rats will freely consume sodium-containing solutions despite a diet surfeit in sodium. In fact, rats prefer dilute hypotonic saline over water and all other NaCl concentrations (Contreras and Smith, 1990). Drawing upon anthropological studies, Denton (1982) suggests that salt taste preference in humans evolved because environmental salt was scarce throughout much of primate and human evolution. By virtue of a largely vegetarian diet, sodium intake was obligatorily low relative to potassium intake and this dietetic pattern favored the development of salt taste preference and salt appetite. Accordingly, salt taste preference reflects the importance of maintaining sodium balance and evolutionarily sodium's relative scarcity in food. In those rare circumstances when salt was available, it was consumed regardless of a physiological need. As a reflection of this legacy, food producers add salt to food during processing to enhance the attractiveness of their product and consumers voluntarily add salt to food beyond that provided naturally. Experimentally, human subjects rate dilute NaCl concentrations as tasting pleasant (Bartoshuk, 1974; Beauchamp and Cowart, 1985; Bertino *et al.*, 1981; 1982; 1983). Sodium depletion enhances salt taste preference and motivates (salt appetite) the organism to seek and exaggerate their consumption of the needed nutrient.

1.2. Gustatory Mechanisms of Salt Intake

Taste is the primary sensory system that functions in the detection, recognition, and ingestion of NaCl. Within the taste system, there appears to be a sodium-sensing module with unique characteristics that may be critical. Electrophysiological studies of single axons or ganglion cell bodies of the salt-sensitive chorda tympani nerve reveal a physiological group of narrowly-tuned neurons that are selectively responsive to NaCl stimuli. These narrowly-tuned, NaCl-specialist neurons respond almost exclusively to salt stimulation, and little, if at all to sweet, sour, or bitter tasting solutions. Among salt stimuli, NaCl-specialists respond specifically to NaCl (and LiCl), but not to KCl and NH_4Cl (Lundy and Contreras, 1998). In contrast, broadly-tuned sodium-responsive neurons respond robustly to all monochloride salts as well as to sour and bitter-tasting solutions (Boudreau *et al.*, 1983; Frank *et al.*, 1983; Lundy and Contreras, 1998). Amiloride, an epithelial sodium channel blocker, suppresses NaCl responses of narrowly-tuned, but not of broadly-tuned, sodium-responsive taste neurons (Ninomiya and Funakoshi, 1988; Hettinger and Frank, 1990; Lundy and Contreras, 1998). It appears that NaCl-specialists neurons are in synaptic contact with taste receptor cells that use an amiloride-

sensitive transduction pathway for sodium detection. Furthermore, anodal electrical stimulation of the tongue is most effective in activating narrowly-tuned salt units with dilute NaCl as the bathing solution. In contrast, broadly-tuned salt units can been driven electrically when either dilute NaCl, KCl, $CaCl_2$, or HCl is in medium bathing the tongue (Ninomiya and Funakoshi, 1988).

There is compelling evidence that the physiological group of NaCl-specialist neurons play a functional role in salt intake behavior of mammals. First, mammals that may be confronted with a low sodium environment, like the herbivorous goat and omnivorous rat, have NaCl-specialist taste neurons (Boudreau *et al.*, 1982; 1985). In contrast, mammals that obtain adequate dietary sodium from meat consumption, like carnivorous dogs and cats, lack a specific sodium-sensing system (Boudreau *et al.*, 1985). Second, the NaCl thresholds of NaCl-specialist neurons are consistent with behavioral recognition thresholds and salivary sodium levels in rats. Their neural response threshold is near 0.03 M NaCl (Frank *et al.*, 1983) corresponding to the lowest concentration recognized as tasting salty (Morrison, 1969; Contreras and Catalanotto, 1980) and slightly above resting salivary sodium level (Contreras and Catalonotto, 1980). Third, neural response discrimination to salt stimulation matches behavioral discrimination patterns. For example, rats can be fooled into consuming the toxic LiCl because of its taste similarity to NaCl (Nachman, 1963), but rats easily discriminate NaCl from KCl (Spector *et al.*, 1996). Fourth, amiloride has been shown to influence a number of salt taste-mediated behaviors in rats and hamsters. For example in rats, amiloride disrupted a conditioned aversion to NaCl (Hill *et al.*, 1990), the expression of depletion-induced salt appetite (Bernstein and Hennessy, 1987; McCutcheon, 1991), the discrimination between NaCl and KCl (Spector *et al.*, 1996), and decreased the unconditioned licking responses to NaCl (Contreras and Studley, 1994). In hamsters, amiloride changed the animals' normal rejection of NaCl in a two-bottle test with water to relative indifference (Hettinger and Frank, 1990).

1.3. Hormonal Mechanism of Salt Intake

With respect to sodium retention, physiochemical changes associated with low body sodium levels activate the renal-renin and blood-angiotensin system to stimulate the release of adrenal-aldosterone, which prevents sodium loss by promoting sodium reabsorption by the kidneys, salivary glands, and sweat glands. Sodium depletion also activates the brain renin-angiotensin system and central angiotensin and aldosterone are hypothesized to act synergistically in the brain to elicit salt appetite (Epstein, 1986). In contrast, excess body sodium suppresses the renin-angiotensin system in the periphery and brain thereby permitting sodium loss and an absence of salt appetite, respectively. In addition, excess sodium and blood volume expansion stimulates the secretion of atrial natriuretic factor from the heart to promote renal sodium excretion (Atlas, 1986; de Bold, 1986). One of the great challenges is to determine how the salt-sensitive taste module is organized and integrated in the central nervous system with hormonal signals also critical for the control of NaCl intake.

The impetus for the present research program comes largely from two lines of research. The first comes primarily from electrophysiological and anatomical studies of the peripheral and central gustatory system underlying salt taste sensitivity. This literature demonstrates that salt taste mechanisms change systematically over the course of development and that they can be modified by dietary experience. The second comes from behavioral and physiological studies linking the NaCl intake of pregnant and lactating rat mothers with changes in the NaCl intakes and blood pressure levels of their adult offspring.

2. SALT TASTE SENSITIVITY MODIFIED BY EXPERIENCE AND DEVELOPMENT

Hill and Mistretta and their colleagues (Hill *et al.*, 1982; 1983; 1987; 1990; This volume) have conducted numerous studies on the development of salt taste sensation. They have shown in both sheep and rats that the peripheral taste nerve responses to NaCl stimulation of the tongue are relatively weak in the earliest stages of development. Through the course of development the peripheral and central taste system became progressively more sensitive to NaCl stimulation as the animals mature. This maturation in sensitivity was due partly to the apparent addition of functional membrane channels sensitive to the sodium transport blocker, amiloride on taste receptor cells (Hill and Bour, 1985). In studies of dietary NaCl experience, Hill and his colleagues have shown that dietary NaCl restriction during the critical period of taste development from embryonic day 3 to at least postnatal day 28 reduced peripheral taste nerve responses to NaCl compared to rats raised on normal NaCl (Hill and Przekop, 1988). This was a temporary effect of NaCl restriction, because soon after placing NaCl-restricted animals on a normal NaCl-containing diet, neural responsivity returned to normal. However, NaCl restriction during prenatal and early postnatal development led to permanent morphological (King and Hill, 1991; 1993) and electrophysiological changes (Vogt and Hill, 1993) in central gustatory neurons.

We set out many years ago to investigate the role of taste in mediating the dramatic shift in behavior from rejection to acceptance of hypertonic saline (salt appetite) after dietary sodium deprivation (Contreras, 1977; Contreras and Frank, 1979) or after sodium depletion caused by adrenalectomy (Kosten and Contreras, 1985). To account for the change in behavior, Richter (1956) proposed a peripheral mechanism suggesting that sodium depletion altered the sensitivity of the taste receptors to NaCl stimulation. In our electrophysiological experiments of taste afferents, we proved Richter right but the direction of the effect was opposite to what Richter expected. Ten days of sodium deprivation (Contreras, 1977; Contreras and Frank, 1979) or adrenalectomy (Kosten and Contreras, 1985) reduced the responses of the chorda tympani to a wide range of suprathreshold NaCl solutions. This reduction was specific to NaCl-specialist neurons as other neuron types were unaffected by sodium deprivation (Contreras and Frank, 1979). Contreras and his associates (1984) concluded that the reversal from rejection to consumption of hypertonic saline was due, in part, to NaCl tasting less intense and more palatable after sodium deprivation. Subsequent electrophysiological studies from sodium-responsive neurons in the first-order sensory nucleus of the brain revealed similar reductions in NaCl responses (Jacob *et al.*, 1988; Nakamura and Norgren, 1995) as was seen in the periphery.

3. SALT PREFERENCE AND BLOOD PRESSURE MODIFIED BYEXPERIENCE

3.1. Early Studies

About 15 years ago, we began our research investigating the relationship between the NaCl intake of pregnant and lactating rat mothers with the NaCl intakes and blood pressures of their offspring. In our first study, adult female rats received a normal diet

of solid food containing either Basal, Intermediate, or High NaCl from conception to postnatal day 30. On postnatal day 30, the offspring from all three salt groups were subsequently maintained on the Intermediate salt diet for two months prior to and during testing. For testing, the adult offspring received a two-bottle, 24-h intake test between deionized water and a NaCl solution. We discovered that adult male and female offspring raised on the High salt diet had a stronger preference for NaCl across a broad range of concentrations than offspring raised on Basal or Intermediate NaCl. Apparently, the saline preference of need-free rats maintained on a surfeit amount of sodium in chow was increased by high maternal NaCl intake. Subsequently, Contreras and Ryan (1990) found that 0.3 M NaCl intake, when it was the sole source of dietary sodium, was linearly related to maternal NaCl exposure. Rats reared on a Basal NaCl diet consumed the least and rats reared on a High NaCl diet consumed the most. In contrast, exposure to a High salt diet later in life, from day 30 onward (Smith, unpublished observations), or to saline from postnatal day 25 to day 65 (Midkiff and Bernstein, 1983) or day 23 to day 60 (Wong, 1980) did not influence later saline preferences. Thus, the work by Contreras and his associates provided evidence suggesting that exposure to a diet rich in salt during an early sensitive period led to a persistent increase in NaCl preference of adult rats.

A parallel line of research investigating the influence of early NaCl intake on adult blood pressure produced a comparable finding (Contreras, 1989; 1993) following the same dietary protocol outlined above for salt preference. In these two studies, blood pressure assessments obtained indirectly by the tail-cuff method over several weeks (Contreras, 1989; 1993) and directly in acute experiments from catheterized carotid or femoral arteries (Contreras, 1989) produced the same general outcome. Adult male and female rats raised on a High salt diet from conception to postnatal day 30 had higher blood pressure levels than those raised on either Intermediate or Basal NaCl. In addition, High salt rats were found to be more responsive to the pressor effect of peripheral angiotensin II and the depressor effect of isoproterenol than rats raised on Intermediate or Basal salt (Contreras, 1989). Peripheral angiotensin II elevates blood pressure directly and indirectly through noradrenergic activation to produce vasoconstriction. Isoproterenol depresses blood pressure by producing vasodilatation in skeletal muscle beds.

Three important aspects of these studies should be appreciated. First, the NaCl levels used in the experimental diets were not too low to be deficient or too high to be toxic and they were within the limits of human NaCl consumption. A young adult rat consumes about 20 g of chow/day (100 kcal/day). On a Basal 0.1% NaCl diet, 0.02 g of NaCl would be ingested. In an adult human consuming 25 times more calories (2,500 kcal/day), the equivalent NaCl intake would be 0.5 g. This would be in the range of a low sodium diet recommended for salt-sensitive, hypertensive individuals who must moderate their NaCl intake. The consumption of the Intermediate 1% and High 3% NaCl diets for rats would be equivalent to the consumption of 5 g (high mid range), and 15 g (high) of NaCl/day for humans. Second, there was an intervening control period during which all the animals in the three maternal NaCl groups were fed the Intermediate salt diet for two months after early salt feeding and during intake and blood pressure testing as well. Therefore the above findings are likely due to the physiological consequences of dietary NaCl that transpire during prenatal or early postnatal development. Third, the above findings appeared in a rat strain with little genetic bias to salt-induced hypertension. High NaCl intake tends to elevates blood pressure only in subjects with a genetic bias to hypertension. Thus the observations by Contreras and his colleagues suggested that a relatively benign dietary manipulation early in development led to an elevation in

salt preference, blood pressure, and blood pressure responsiveness in adult normotensive Sprague-Dawley rats.

In summary, considerable literature exists on the role of dietary NaCl on taste and salt intake as well as on blood pressure regulation of adults. Recent studies indicate, however, that NaCl levels in the diet may have its most profound effect early in ontogenesis when there are important developmental changes in the neuroendocrine mechanisms that control sodium and blood pressure regulation. These neuroendocrine mechanisms appear to be plastic subject to modification by dietary experience particularly during periods of rapid developmental change. Enhanced or restricted experience with dietary NaCl through the maternal diet can influence the offsprings' NaCl intake, blood pressure, and sodium taste system. The aims of our multiproject research program are to examine the consequences and mechanisms of early salt intake on taste and blood pressure. We have emphasized the integration of behavioral, physiological, morphological, and neurobiological approaches using Sprague-Dawley rats, an invaluable model for the study of neuroendocrine mechanisms of water and electrolyte balance and cardiovascular regulation.

In this review, I will summarize the current work by Contreras and Smith characterizing the effects of maternal NaCl intake on the offspring's taste and blood pressure reactivity to NaCl. This review will also include the current work from Spector's lab using sophisticated psychophysical procedures to examine changes in taste thresholds in rats raised on different maternal NaCl diets. And finally, the current work from Rowland's and Katovich's lab examining various hormonal and neurochemical mechanisms that may underlie changes in blood pressure and salt intake behavior due to early dietary NaCl will be summarized. A major focus here is on the renin-angiotensin system. In short, this paper will summarize basic new information on the consequences and mechanisms of early exposure to known amounts of NaCl on taste and blood pressure in an important animal model.

3.2. Methodological Considerations

There were several methodological concerns of the earlier research that required attention and changes in our general protocol for raising the animals for the multiproject program. In the earlier research (Contreras and Kosten, 1983; Contreras, 1989; 1993), the foundation diet used during early exposure and that used after postnatal day 30 differed perhaps in an important way. Previously, female rats and their offspring were fed a special test diet (ICN Pharmaceuticals) until postnatal day 30, after which they were switched to commercial rat pellets. Both diets were similar in their proportional fat, protein, and carbohydrate content, but the carbohydrate source differed between the two. The test diet consisted of sucrose (65% by weight), while standard rat chow consists of starch. In fact, the total sugar content of commercial chow is less than 6% (sucrose = 3.7%). It is unknown whether this difference in carbohydrate source from sugar during the perinatal exposure period to starch from postnatal day 30 onward contributed to the differences seen in solution preference and blood pressure. Nevertheless, this was a factor in the earlier research.

Another concern was that prior research used relatively few (e.g., three) litters in each dietary salt condition and littermates were considered independent subjects in testing and statistical analyses (Contreras and Kosten, 1983; Contreras and Ryan, 1990). This design opens the possibility that common genetic background and/or identical intrauterine and postnatal environments may have contributed to the results. Also, many

of the adult animals used for breeding were likely siblings. When purchasing rats of specific age and weight range, commercial suppliers will typically, as we have recently discovered, send animals to labs derived from few litters, often siblings, unless instructed otherwise. Thus, it is likely that brother-sister matings were responsible for producing some of the experimental offspring of earlier research. Consequently, inbreeding may have compromised the results of prior research. Because there are both genetically-mediated strain differences in salt taste preference in laboratory rats (Midkiff *et al.*, 1985) and mice (Beauchamp and Fisher, 1993; Gannon and Contreras, 1995), and within-strain differences in NaCl-induced hypertension (Oparil *et al.*, 1988), it is extremely important to obtain subjects with broad genetic heterogeneity.

These concerns associated with the foundation diet and breeding were eliminated from the experimental design of our multiproject research program. The foundation diet stayed the same throughout the research consisting of starch as the carbohydrate during early exposure as well as during adult testing; the only dietary parameter to vary was NaCl content. Second, we advanced genetic heterogeneity of our test offspring by using a nonsibling-derived breeding stock. In addition, several litters were represented in each group typically with only one male offspring per litter being used as a subject for a given experiment.

To adapt them to their experimental diet, adult female rats began receiving one of the NaCl-containing diets (0.1, 1, 3, or 6 %) five days before mating. They continued to receive their respective NaCl diet throughout pregnancy and lactation to postnatal day 30. Within 24–48 hours of birth, each litter was culled to eight, retaining as many male pups as possible, and weaned on postnatal day (PD) 21. At PD 30, all the animals from each salt condition began receiving the Intermediate 1% NaCl diet until testing as adults between 90–120 days of age.

In prior research, we have used a broad 30-fold range of dietary NaCl levels, not too low to be deficient or too high to be harmful or toxic. For example using the sugar-based foundation diet, we have found that mother rats on either 0.1, 1, or 3% salt diets gave birth to normal offspring in terms of litter size and pups' birth weights and plasma Na^+ and K^+ concentration (Contreras, 1989). In our current research using the starch-based foundation diet, we have recently extended the range of NaCl levels and added a fourth salt group (Super High) raised on 6% NaCl. We have several observations of the mothers and their offspring particularly during the exposure period. There were several interesting effects and noneffects of dietary NaCl.

3.3. Food Intake and Birth Weight

With respect to the mothers, their food intake was surprisingly stable despite a 60-fold difference in dietary NaCl level (Snyder *et al.*, 1998). As shown below, the average food intake during the 21-day pregnancy and lactation periods was similar for the four salt groups. The heightened food intake during lactation in comparison to gestation, was likely due to the mothers' enhanced energy requirement and the pups consumption of food beginning around PD14. A similar food intake among all four groups translates into a proportional difference in NaCl intake related to its level in the diet. This obligatory difference in NaCl ingestion was accompanied, as can be seen below, by a small increase in water intake during gestation and a big increase during lactation as the water intake of the High and Super High groups was greater than that of the Basal and Intermediate salt groups. Furthermore, Bird and Contreras (1986) have shown that pregnant rats can maintain sodium balance by compensatory changes in urinary sodium excretion

Table 1. Average food and water intake during gestation and lactation of mothers fed Basal 0.1, Intermediate 1, High 3, or Super High 6% NaCl

VARIABLE	Basal	Intermediate	High	Super High
Gestation				
Food intake, g	24.7 ± 2.1	24.3 ± 1.5	24.3 ± 2.6	26.1 ± 3.3
Gestation				
Water intake, ml	73.9 ± 13.0	62.8 ± 12.8	85.4 ± 16.5	95.2 ± 9.5
Lactation				
Food intake, g	48.6 ± 1.0	51.9 ± 1.7	51.6 ± 1.3	53.1 ± 1.4
Lactation				
Water Intake, ml	71.9 ± 6.9	78.6 ± 4.6	120.1 ± 9.5	176.9 ± 2.8

while being fed the sugar-based Basal, Intermediate, or High NaCl diet. Pregnant females on the Basal salt diet excreted low amounts of sodium, while females on High salt excreted high amounts of sodium relative to females fed Intermediate salt. We have not yet examined sodium excretion during lactation, nor have we examined the effect of 6% NaCl on electrolyte excretion at any point during pregnancy or lactation.

With respect to the offspring, we have bred about 50% fewer mothers on the Super High NaCl diet than on the other three salt diets. Nevertheless, there are considerable data from which to compare. In general, the pups were born healthy and were well cared for by their dams throughout lactation. The birth weights shown below compares favorably to that published previously using the sugar-based NaCl diet (Contreras, 1989). However, litter size has increased over the past 12 years averaging about 12 pups/litter in 1986 (Bird and Contreras, 1986) and 14 in 1989 (Contreras, 1989) on the sugar-based diet, to about 15–16 currently on the starch-based diet. It is unlikely that carbohydrate (sugar or starch) plays a role in this upward trend. It is more likely due to the effects of selective breeding leading to a progressive generational change in the biological capacity of Spraque-Dawley rats to produce offspring.

As shown in Table 2, dietary NaCl level influenced birth weight. The average weight of Basal pups was one gram less than the weight of pups from the other three salt groups, which were similar. This early weight effect had persistent consequences to adulthood. Body weight measures from weaning to adulthood showed that the four salt groups had normal growth curves. However, the Basal group typically weighed less than the other three groups throughout testing. In adulthood, the Intermediate group weighed the most, and the High and Super High groups weighed an intermediate amount between the Intermediate and Basal groups.

3.4. Hormonal Effects

Rowland and his colleagues (Crews *et al.*, 1997) have recently examined the hormonal consequences of maternal dietary NaCl on dams and their offspring. They have

Table 2. Average birth characteristics of 1-day-old rat pups from mothers fed Basal 0.1, Mid 1, High 3, or Super High 6% NaCl

VARIABLE	Basal	Intermediate	High	Super High
Number of litters	80	84	70	43
Pups/litter	14.9	15.7	15.8	15.7
Birth weight, g	5.8	6.8	6.8	7.0

assessed plasma aldosterone and plasma renin activity in nonlactating and lactating female rats fed Basal, Intermediate, or High NaCl. By far the most dramatic effects were in plasma aldosterone, but similar effects emerged in plasma renin activity, too. In nonlactating rats, the plasma levels of both hormones were highest in the Basal group and declined progressively with NaCl level. For example, the plasma aldosterone level of the Basal group was two-fold higher than that of the Intermediate group and four-fold higher than that of the High group. Lactation greatly exaggerated these differences by increasing the aldosterone level of just the Basal group from about 450 pg/ml in nonlatating females to about 3800 pg/ml in lactating females. The aldosterone levels were similar in lactating and nonlactating females of about 250 pg/ml in the Intermediate NaCl group and about 150 pg/ml in the High NaCl group. These data may suggest that the greatest impact of dietary NaCl is on the dams fed Basal NaCl and not High NaCl. However, it is perhaps more likely that the magnitude of the directional effect from the Intermediate salt level represents a floor effect in suppressing the salt-retaining hormones with excess dietary NaCl, and a relatively open range in stimulating hormone release with low NaCl. Plasma aldosterone levels have not been assessed in lactating females fed Super High 6% NaCl.

Plasma aldosterone and plasma renin activity have also been measured in Basal, Intermediate, and High salt offspring on PD21 prior to weaning, and PD28 after a week on their respective salt diet without their mother (Rowland, Crews, and Aerni, unpublished observations). Like the data from dams, the hormone levels from Basal offspring were relatively conspicuous, particularly on PD28. At both ages, both hormones were lowest in pups fed High NaCl and highest fed Basal NaCl. In Basal offspring, the levels of both hormones were three-fold higher on PD28 than on PD21. This observation suggests that dietary NaCl may have a greater effect on the offspring after than before weaning.

3.5. Effects on Salt Preference

Contreras and Kosten's (1983) original report demonstrating the effect of early NaCl exposure on salt preference has never been replicated. In their original study, Contreras and Kosten (1983) examined the influence of maternal NaCl exposure on the offspring's preference for NaCl, KCl, and glucose in a two-bottle test with water over 48 hours. They found no group differences in KCl taste preference, which indicates a relatively specific effect on sodium salts and not to other cations. However, male rats exposed perinatally to high dietary NaCl exhibited a stronger glucose preference than rats raised on intermediate or Basal NaCl; this effect was not seen in females. Subsequently, adult offspring with high NaCl experience were found to consume greater amounts of sweetened condensed milk than those reared with Basal NaCl (Contreras, 1993). These results raise the possibility that early experience with a High NaCl diet elevates sweet taste preferences, or that increased NaCl preference generalizes to sweet-tasting compounds.

We have reexamined the effect of early NaCl exposure on NaCl, KCl, and glucose solution intake (Snyder *et al.*, 1997). In general, we modeled our experiments exactly after the original work conducted 15 years ago (Contreras and Kosten, 1983), except that we eliminated the methodological concerns cited previously. We have also recently added a super-High salt group exposed perinatally to 6% NaCl. Figure 1 below shows the average salt preference (salt intake/total fluid intake $\times$ 100) of the four groups given a two-bottle test between water and 0.3 M NaCl for seven consecutive days. As can be seen, the average salt preference of adult rats varies systematically with maternal NaCl intake. Adult rats

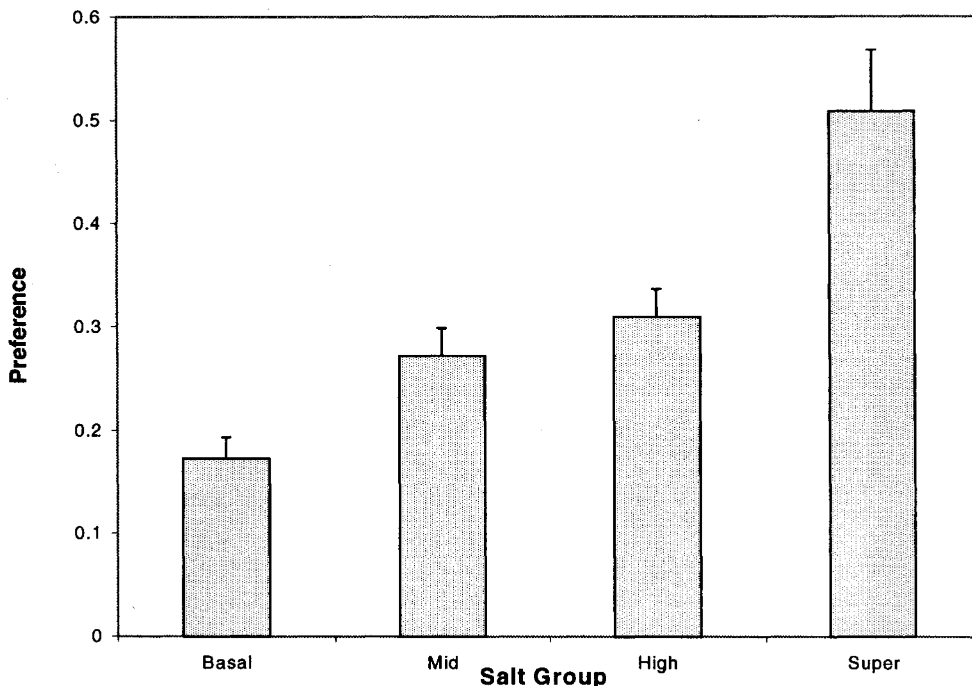

Figure 1. The average 0.3 M NaCl preference of adult rats raised perinatally on a chow diet containing either Basal 0.1%, Mid 1%, High 3%, or Super High 6% NaCl.

raised on Basal NaCl had the lowest salt preference and rats raised on Super High NaCl had the highest salt preference (Snyder *et al.*, 1998). The other two groups had an intermediate salt preference.

It should be noted that there was sizeable within-group and between-group variation. We began to see a consistent difference in salt preference only after repeated testing with a relatively strong salt concentration, not with a range of NaCl concentrations tested over shorter periods. Furthermore, KCl and glucose preference also seems to be elevated by high maternal NaCl intake, although not to the same degree as with NaCl. Regardless of these considerations, the present results provide additional evidence linking the NaCl intakes of pregnant and lactating mothers with the solution intakes of their offspring.

3.6. Taste Psychophysics

Spector and his colleagues have recently explored the possibility that the difference in NaCl solution preference may be due to a difference in taste assessed psychophysically in brief access experiments. In the first experiment, NaCl detection thresholds were determined in adult rats raised on a maternal diet of either Basal, Intermediate, or High NaCl (Geran and Spector, 1998). Thirsty rats were trained to press one lever if they tasted a NaCl solution and a second lever if they tasted water. Percent correct responses were measured across several NaCl concentrations. In a second experiment, licking responses were measured to brief presentations of a wide range of suprathreshold NaCl concentrations (Sauer and Spector, 1998). A second aspect of these experiments was to deter-

mine whether 100 uM amiloride, when added to the NaCl solution, differentially altered thresholds and the NaCl concentration-lick rate functions of Basal, Intermediate, and High salt rats.

The results from Spector's experiments are inconsistent with a taste explanation for the difference in salt preference between Basal, Intermediate, and High salt rats. First, Geran and Spector (1998) found that the NaCl detection threshold of Basal, Intermediate, and High salt rats was similar averaging 0.005 M. Amiloride increased NaCl thresholds slightly to 0.036 M for all groups. Second, the NaCl concentration-lick rate functions were also the same for the three groups of salt-exposed rats (Sauer and Spector, 1998). At the weakest NaCl concentration, lick rate was at its peak and declined progressively with increasing NaCl concentration. Amiloride shifted the descending lick rate functions to the right by about one-tenth of a log unit for all three groups. NaCl detection thresholds and lick rate functions were also unaffected by perinatal exposure to Super High 6% NaCl or by maintaining the animals on their respective NaCl diets throughout life and not restricting it to 30 days after birth.

Taste may not, however, be entirely eliminated as a mechanism by these psychophysical results. Taste differences may not reveal themselves particularly when the animals are severely challenged by water deprivation, they are highly motivated and the cost is high for poor performance, and there are compensatory mechanisms to offset a taste difference. Even in the limited behavioral context of a psychophysical experiment with brief, 20-s periods of stimulus availability, all of the sensory modalities of the oral cavity and possibly post-ingestion cues also may be recruited in the performance of a highly motivated animal. Just with respect to taste, there are four nerve branches from three cranial nerves to help the animals make the discrimination. They need not rely solely on the salt-sensitive chorda tympani nerve to make the discrimination.

3.7. Taste Physiology

We have recently (Pittman and Contreras, 1998) recorded the integrated responses of the whole chorda tympani nerve to a range of NaCl, KCl, and quinine hydrochloride concentrations alone and mixed with 100 uM amiloride in adult rats raised perinatally on Basal, Intermediate, or High NaCl. We found that the neural concentration-response functions for the three stimuli without amiloride were unaffected by maternal NaCl intake. For all three groups, amiloride suppressed NaCl responses twice as much as KCl responses, but amiloride had no effect on quinine responses (see Fig. 2). The three salt groups differed in amiloride suppression of NaCl responses, but not of KCl responses. Amiloride suppression was greater in the High salt group than in the Intermediate or Basal salt groups, which were the same. These results suggest that perinatal high salt intake may up-regulate the number of amiloride-sensitive channels on taste receptors innervated by the chorda tympani nerve. Thus, taste may play a role in mediating the elevated salt preferences of rats raised perinatally on High NaCl. Additional electrophysiological and behavioral experiments will be conducted to determine whether this finding holds up and its relevance demonstrated behaviorally.

3.8. Blood Pressure

We have re-investigated the long-term influence of perinatal salt exposure on the blood pressure and heart rate of adult rats (Wong, Wilson, Henderson, and Contreras, 1998). In our prior studies, blood pressure and heart rate measures were taken by

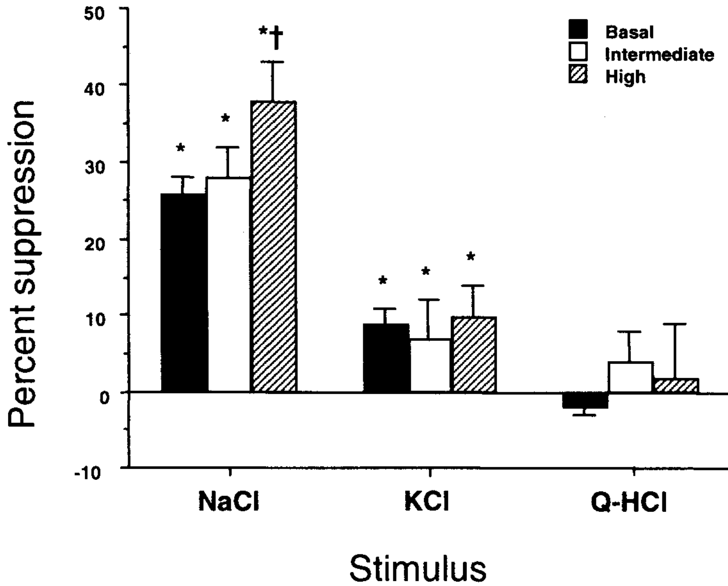

Figure 2. The percent amiloride suppression of the whole chorda tympani nerve in response to NaCl, KCl, and quinine HCl in adult male rats raised perinatally on either Basal 0.1%, Intermediate 1.0%, or High 3% NaCl.

tail-cuff in anesthetized (Contreras, 1989) or restrained rats (Contreras, 1993) or by arterial catheterization in tethered animals (Contreras, 1989). While its noninvasiveness is an advantage, the tail-cuff method is disadvantageous because the measure is indirect, discontinuous, and can only be obtained after restraint or anesthetization, and often the animal must be warmed to stimulate vasodilatation and detect pulses from the tail artery. Catheterization eliminates many of these measurement concerns. Nevertheless, there are inherent problems with tethering in terms of restricting animal mobility and the challenge of maintaining a viable tubular connection linking the animal's vascular system with an external transducer, which limits the time over which blood pressure assessments can be obtained. Recording blood pressure by telemetry overcomes the limitations of other methods. Using Data Sciences' pressure transmitters and receivers in conjunction with our own data collection system, we have measured mean arterial pressure (MAP) and heart rate (HR) continuously for several weeks from the descending aorta of 21 adult male offspring: 8 High salt rats, 6 Intermediate salt rats, and 8 Basal salt rats. Throughout testing, food and deionized water were available at all times while on a 12:12 light/dark cycle.

In general, MAP and HR were relatively low and stable during the light phase, compared to higher and more variable levels during the dark phase. The average MAP of the High salt group was higher in both the light and dark (L = 102 mm Hg, D = 108 mm Hg) than the MAP of the Intermediate (L = 92 mm Hg, D = 98 mm Hg) and the Basal salt group (L = 88 mm Hg, D = 93 mm Hg). The average HR changed more dramatically from light to dark for all the three salt groups. However, the average HR in the light and dark (L = 300 beats/min; D = 359 beats/min) of the High salt group was similar to that of the Intermediate (L = 295 beats/min; D = 359 beats/min) and Basal salt group (L = 295 beats/min, D = 350 beats/min). The average MAP over the circadian cycle is shown in Fig. 3 for the three salt groups while being maintained on the Intermediate salt diet; the

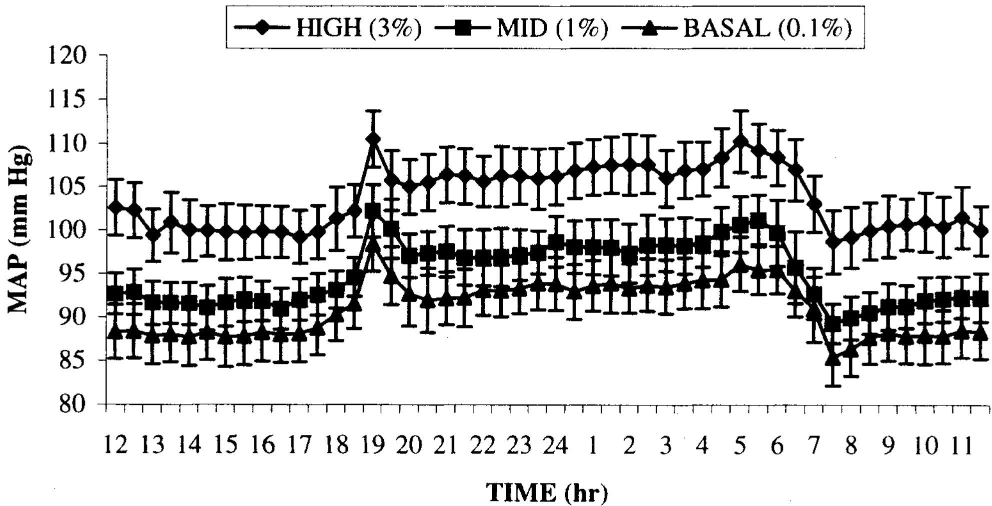

Figure 3. The average mean arterial pressure (MAP) over a 24-hour period in adult rats raised on either Basal, Mid, or High NaCl from conception to postnatal day 30.

animals were in the dark from 7 PM (19) to 7 AM (7). Interestingly, the absolute MAP levels of the three salt groups were unchanged when switched to a High 3% salt diet for two weeks. Thus in this limited context, High perinatal NaCl intake produced a long-term elevation in maintained MAP level, without influencing blood pressure reactivity to a High salt diet in adulthood. That is, high salt intake in adulthood did not enlarge on the blood pressure effect of high perinatal NaCl intake. In future experiments, we plan on challenging the animals with a higher NaCl level for a longer period of time to assess further the influence of perinatal NaCl intake on blood pressure reactivity to a high salt diet. It is important to know for certain that NaCl can permanently scale MAP early in life and not alter the MAP response later in life. The mechanisms affected by dietary NaCl early in development may be different from those used in adulthood to govern blood pressure response to dietary salt.

3.9. Angiotensin II Receptors: A Possible Mediator

Others and we have explored the possibility that the difference MAP among High, Intermediate, and Basal salt rats may be mediated by changes in angiotensin II receptors in the brain. Moe (1987) used autoradiographic ligand binding to examine angiotensin II receptor density and affinity in the brains of adult rats raised on a maternal diet of either 0.1, 1, or 3% NaCl following the same protocol used in our research. She discovered that maternal NaCl level correlated positively with receptor density in hypothalamic tissue around the third ventricle, and correlated negatively with receptor density in brainstem tissue around the fourth ventricle. The opposite pattern of results was found for angiotensin II receptor affinity. The brain areas where maternal NaCl influenced angiotensin II receptors contain circumventricular organs involved in mediating the pressor and water intake responses to peripheral angiotensin II (Barnes and Ferrario, 1987; Reid, 1984).

Preliminary findings from two behavioral studies are consistent with Moe's (1987) radioligand study. First, Aerni, Rowland, and Katovich (1998) have reported that the

one-hour water intake after peripheral angiotensin II (200 ug/kg body weight) was greater in adult rats raised on High NaCl (7.6 ± 0.8 ml) than in rats raised on Intermediate (4.4 ± 1.2 ml) or Basal (3.5 ± 1.3 ml) NaCl. The second experiment consisted of 8 NaCl groups. Sprague-Dawley dams and their litters were fed either the Basal or High NaCl diet. Two groups were fed either the Basal or High NaCl diet from conception to postnatal day 30 (Groups BBB and HHH). Three groups were fed the High NaCl diet restricted to either the 21 days of gestation, postnatal days 1–15, or postnatal days 16–30 (Groups HBB, BHB, BBH). Because the sensitive period for NaCl may overlap across these three exposure periods, the last three groups were fed the High NaCl diet during two of them (Groups HHB, BHH, HBH). When not on the High NaCl diet, the animals were fed the Basal NaCl diet and beyond postnatal day 30 until testing. To minimize any physiological adaptations that may accompany switching abruptly from one NaCl level to the other, the dams were maintained on the same NaCl diet throughout pregnancy to postnatal day 15 and switched only then. All litters were cross-fostered within 24-h of birth to a dam on the appropriate dietary NaCl level. Beginning at 90 days of age, the rats were examined for differences in angiotensin II-elicited water intake after 2 μl infusions of 100 nM angiotensin II into the lateral ventricle and water intake was measured over the ensuing 20 min.

Figure 4 shows the data from the two extreme conditions of either High NaCl (HHH) or Basal NaCl through all three exposure periods. As can seen, animals raised on High NaCl drank twice as much water in response to angiotensin II as those raised on Basal NaCl. Figure 5 shows the results from animals raised on High NaCl during one of three exposure periods. The results from the HHH and BBB groups are re-plotted for comparison. As indicated below, the group raised on High NaCl from postnatal 15–30

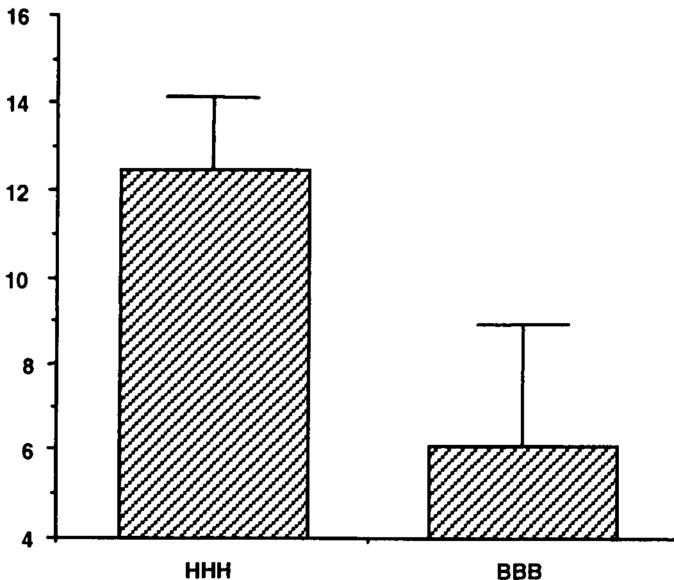

Figure 4. The average water intake to intraventricular administration of angiotensin II in adult rats raised on either a Basal 0.1% (BBB) or a High 3% (HHH) NaCl diet from conception to postnatal day 30.

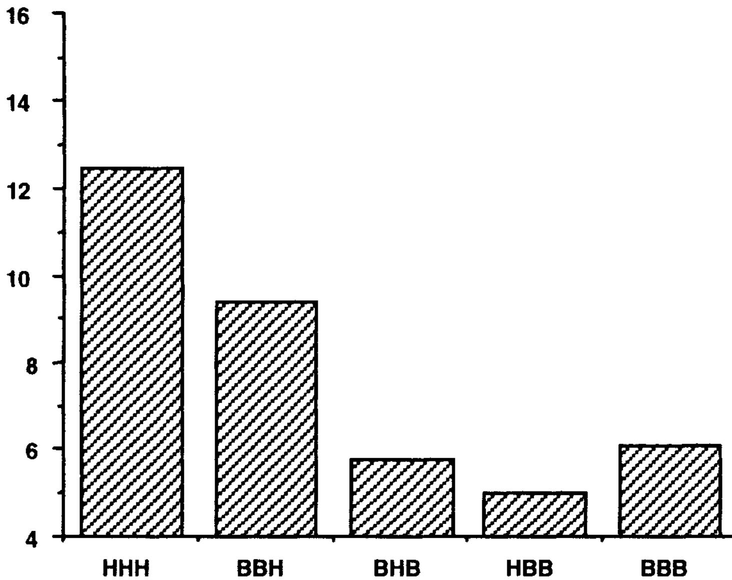

Figure 5. The average water intake to intraventricular administration of angiotensin II in adult rats raised on a High NaCl diet restricted to gestation (HBB), postnatal days 1–15 (BHB), or to postnatal days 16–30 (BBH).

(BBH) was most like the HHH group in terms of water intake. The other two groups' (BHB, HBB) water intake was similar to the intake of the BBB group.

In comparison to these data, the overall water intake of animals raised on High NaCl during two of the three exposure periods was generally greater than that of animals raised on High NaCl during just one exposure period (see Fig. 6). Group BHH consumed the most water in comparison to the other groups. These results suggest that with respect to angiotensin II-elicited water intake, the sensitive period for maternal NaCl intake, may be after birth either during lactation or the early post-weaning period (see BBH and BHH). Additionally, angiotensin II receptor function may be up-regulated by high maternal NaCl intake.

4. SUMMARY AND CONCLUSION

Our research has produced evidence linking the NaCl intake of expectant and lactating rat mothers with the blood pressure and NaCl intake of their offspring as adults. Our working hypothesis is that at some critical point in the offspring's' early development, animals exposed directly or indirectly to low or high NaCl may experience a permanent, organizational change in the neuroendocrine machinery that controls blood pressure and NaCl intake. In particular, changes in sodium-specific ion channels on taste receptors and angiotensin II receptors in the brain may underlie in part changes in NaCl intake and blood pressure, respectively.

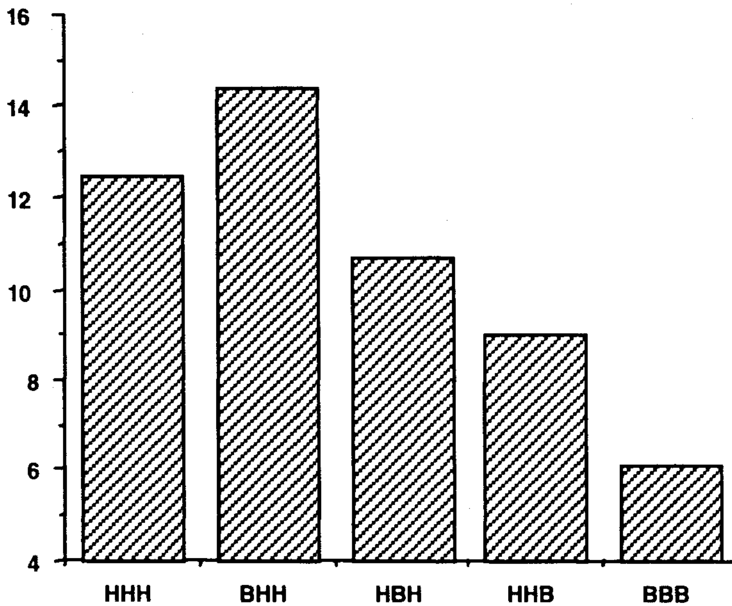

Figure 6. The average water intake to intraventricular administration of angiotensin II in adult rats raised on a High NaCl diet from conception to postnatal day 15 (HHB), from birth to postnatal day 30 (BHH), or during pregnancy and from postnatal day 16–30 (HBH).

5. ACKNOWLEDGMENTS

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5

THE ROLE OF EARLY EXPERIENCE IN OLFACTORY BULB CELL SURVIVAL

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1. DECREASED EARLY OLFACTORY EXPERIENCE

1.1. Restricted Early Experience Decreases Neuronal Number in the Olfactory Bulb

Restriction of early sensory experience alters brain development, but the consequences of reduced experience are particularly dramatic in the olfactory system. In other sensory systems, restricted early experience has not been shown to cause cell death, unless the system has been physically damaged or activity in the sensory nerve has been suppressed with tetrodotoxin (Born and Rubel, 1988; Catsicas *et al.*, 1992; Galli-Resta *et al.*, 1993; Salvi and Henderson, 1996; Rauschecker, 1991; Woolsey, 1990). In the bulb, however, physical closure of an external naris on postnatal day (P) 1 or 2 restricts early olfactory stimulation and thereby reduces olfactory bulb neuronal activity (Brunjes, 1994). By P20–30, the density of tufted cells, glial cells, juxtaglomerular cells and granule cells decrease by 30–45% (Meisami and Safari, 1981; Skeen *et al.*, 1985; Frazier and Brunjes, 1988). The decreased cell number in the odor-restricted bulb is due to cell death, rather than to a decreased rate of neurogenesis (Frazier-Cierpial and Brunjes, 1989).

1.2. Odor Restriction Increases DNA Fragmentation in the Olfactory Bulb

Cells containing fragmented DNA can be labeled *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) (Gavrieli *et al.*, 1992). When this assay was applied to 20-μm sections from unmanipulated rats at P15, isolated cells were labeled throughout the olfactory bulb, suggesting a significant naturally occurring level of DNA damage in the developing bulb (Najbauer and Leon, 1995). The density of labeled cells was similar in all analyzed layers of the main olfactory bulb (5–8 cells/mm^2). After naris closure was performed on P2, the density of TUNEL(+) cells

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increased significantly in all of these laminae on P15. There was a high level of TUNEL positive cells in the normal developing accessory olfactory bulb, but since naris occlusion would not be expected to restrict sensory cues to this system, we expected and found no additional increase in DNA fragmentation in that structure.

1.3. There May be Compensatory Responses in Odor-Restricted Bulbs That Diminish the Functional Consequences of Reduced Early Experience

Despite the obvious damaging effects of naris closure on the olfactory bulb (Brunjes and Frazier, 1986; Brunjes, 1994), it is remarkable that considerable structure and function are maintained after restricted early experience. Indeed, most bulb neurons survive in the absence of normal odor stimulation (Leon, 1999). We also found that the odor-restricted rat bulb maintains the ability to respond fairly well to odors. Spatial patterns of glomerular 2-deoxyglucose (2DG) uptake, as well as relative quantitative uptake of 2DG were no different in odor-restricted and normal bulbs. Moreover, the ratio of excitatory to inhibitory responses by individual mitral cells in odor-restricted bulbs following odor stimulation through the reopened naris (Guthrie *et al.*, 1990; Wilson *et al.*, 1990) is similar to that observed in the normal bulb. We did find some differences in the odor-restricted bulb, namely an increased size of 2DG uptake foci and increases in the number of responsive mitral cells (Guthrie *et al.*, 1990; Wilson *et al.*, 1990), but these changes did not suggest a highly damaged system. Even on a behavioral level, there is little decrement in odor responses. Odor-restricted infant rabbits who have their nares reopened, and their normal bulb ablated, have the normal attraction to the mother's nipple pheromone (Stahl *et al.*, 1990).

It seemed possible that the loss of olfactory stimulation is accompanied by a commensurate change in the olfactory nervous system that allows function to be maintained. In an example of what may be a compensatory response, the transient increase in norepinephrine in the odor-restricted rat bulb (Brunjes *et al.*, 1985) is followed by a decrease in beta-adrenergic receptors (Woo and Leon, 1995). In what also may be a compensatory effect, we reported that the decreased dopamine levels that are characteristic of early odor restriction (Brunjes *et al.*, 1985) are associated with increases in D2 dopamine receptors (Guthrie *et al.*, 1991). We also reported that the number of low-affinity NGF receptors increases in the glomerular layer of the odor-restricted bulb (Gomez-Pinilla *et al.*, 1989), a response that also may be a compensation for the loss of early neural activity.

Although there are a variety of possible explanations for each of these findings, they may all indicate compensatory mechanisms by which an odor-restricted bulb minimizes the impact of decreased neural activity (Leon, 1999). The bulb may have evolved these mechanisms in response to the naturally occurring odor restriction caused by nasal infections (Leon, 1999), allowing fairly normal development to occur in the face of reduced activity.

1.4. Odor Restriction Increases the Expression of the Anti-Death Gene, *bcl-2*

In addition to causing increased DNA fragmentation, naris closure also increased levels of mRNA for *bcl-2*, a gene that can prevent cell death (Reed, 1994), in the odor-restricted bulb (Najbauer *et al.*, 1995). An increase in bcl-2 expression was detected in cells across the entire bulb when visualized with *in situ* hybridization, and the increase was present across all laminae of the lateral bulb. The changes in *bcl-2* mRNA in the lateral glomerular layer were inversely correlated with changes in the number of

TUNEL(+) cells in the glomerular layer as measured in alternate sections from the same brains (Najbauer *et al.*, 1995), data consistent with the notion that Bcl-2 may suppress cell death.

1.5. How May Bcl-2 Compensate for the Loss of Normal Olfactory Stimulation?

Bcl-2 protein can suppress cell death (Reed, 1994). Forced expression of *bcl-2* in cultured mammalian cells prevents cell death caused by a number of agents, suggesting that it can act at a convergent step downstream from multiple pathways that lead to cell death (Garcia *et al.*, 1992; Allsopp *et al.*, 1993; Kane *et al.*, 1993; Mah *et al.*, 1993; Myers *et al.*, 1995; Zhong *et al.*, 1993). Transgenic mice that overexpress human *bcl-2* in neurons lack the normally occurring developmental neuronal death throughout the brain (Martinou *et al.*, 1994; Farlie *et al.*, 1995; Burne *et al.*, 1996; Zanjani *et al.*, 1996). Loss of neurons caused by axotomy also is blocked in mice with elevated brain *bcl-2* expression (Dubois-Dauphin *et al.*, 1994; Farlie *et al.*, 1995; De Bilbao and Dubois-Dauphin, 1996; Burne *et al.*, 1996). Conversely, transgenic mice that are deficient in *bcl-2* display neuronal death that extends beyond the normal phase of developmental cell death in certain regions (Michaelidis *et al.*, 1996). Neurons cultured from *bcl-2* deficient mice also display an increased probability of dying in response to growth factor withdrawal (Greenlund *et al.*, 1995).

1.6. Bcl-2 Family Members Either Can Promote or Prevent Cell Death

Bcl-2 is only one of a set of homologous proteins that can suppress cell death (Reed *et al.*, 1996). This family includes Bcl-x_L (Boise *et al.*, 1993), which is abundant in the olfactory bulb (Frankowski *et al.*, 1995; Alonso *et al.*, 1997), as is Bcl-2 itself (Castrén, *et al.*, 1994; Merry *et al.*, 1994). Other death-suppressing members of this family include A1 (Lin *et al.*, 1993), Mcl-1 (Kozopas *et al.*, 1993), and Bcl-w (Gibson *et al.*, 1996), which have not been studied in the olfactory bulb. Other proteins showing homology to Bcl-2 act as promoters of cell death. The best characterized of these is Bax (Oltvai *et al.*, 1993), homodimers of which appear to be the active effectors of cell death in several experimental models (Reed *et al.*, 1996), although monomeric forms also may promote cell death (Simonian *et al.*, 1996). Transgenic mice deficient in Bax undergo less programmed cell death in certain brain regions, and these neurons do not die even in response to axotomy (Deckwerth *et al.*, 1996). The death-suppressing members of the Bcl-2 family generally are thought to form heterodimers with Bax and thereby inactivate its death-inducing function (Oltvai *et al.*, 1993; Sato *et al.*, 1994; Sedlak *et al.*, 1995; Reed *et al.*, 1996). However, some mutants that are incapable of binding Bax have been shown to reduce cell death, suggesting an active death-repressing activity for their homodimers (Cheng *et al.*, 1996). Transgenic mice lacking Bcl-x undergo massive embryonic neuronal cell death (Motoyoma *et al.*, 1995), but if these mice are also deficient in Bax, this additional cell death does not occur (Shindler *et al.*, 1997). These data argue for the importance of interactions between Bax and Bcl-x_L. Other death-promoting members of the family include Bad (Yang *et al.*, 1995), Bcl-x_S (Boise *et al.*, 1993), and Bak (Chittenden *et al.*, 1995; Farrow *et al.*, 1995; Kiefer *et al.*, 1995), which also are capable of binding to the death-suppressing members, and may displace them either from homodimeric complexes, or from heterodimers with Bax (Reed *et al.*, 1996). To our knowledge, none of the death-promoting members of the family have been investigated in the olfactory bulb,

although Bax and Bak have been found to be associated with dying neurons in other brain areas (Krajewski *et al.*, 1994; 1996).

1.7. Expression of the bcl-2 Gene Family is a Regulated Process

If the members of this gene family are constitutively expressed in cells, regardless of the activity of the cell, then one would not expect that experience of any kind would affect their expression. If, to the contrary, the genes were regulated by neural activity, they could serve as a potential mechanism for the survival of cells following changes in early experience. Indeed, the promoter regions of the *bcl-2* and *bax* genes contain sequences that suggest their regulation by other proteins. For example, the transcription of *bcl-2* can be decreased by the tumor suppressor p53 (Miyashita *et al.*, 1994a,b), which is a direct activator of *bax* expression (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994a; Miyashita and Reed, 1995). The *bax* promoter sequence also contains elements suggesting possible regulation by *myc/max* and by redox status (Miyashita and Reed, 1995). In addition, growth factors can stimulate the transcription of *bcl-2* and *bcl-*x_L in cultured cells (Hanada *et al.*, 1993; Bullock and Johnson, 1996; Katoh *et al.*, 1996; Zhai *et al.*, 1996; Parrizas and Leroith, 1997).

Upregulation of bcl-2 gene family members also has been shown *in vivo* in the nervous system. For example, a brief ischemic event produces a long-lasting upregulation of Bcl-2 and protects against a later, prolonged ischemia that normally would induce hippocampal cell death (Shimazaki *et al.*, 1994). Similarly, brief ischemia induces the upregulation of Bcl-2 and does not cause death of cortical neurons (Chen *et al.*, 1995). In contrast, either prolonged ischemia, excitotoxin administration or axotomy induce Bax and neuronal death (Gillardon *et al.*, 1995; Gillardon *et al.*, 1996; Hughes *et al.*, 1996; Krajewski *et al.*, 1995). Thus, there are several precedents for regulation of these genes and proteins that lend plausibility to the hypothesis that the olfactory bulb may be able to compensate for odor restriction by upregulating cell death suppressor genes. At the same time, upregulation of death-promoting genes may account for the increased cell death after early olfactory restriction.

1.8. Bcl-2 May Rescue Olfactory Bulb Cells at Risk of Dying

We have found increased DNA fragmentation in the cells of odor-restricted bulbs (Najbauer *et al.*, 1995). However, DNA fragmentation does not always lead to cell death (Didenko and Hornsby, 1996). In the presence of high levels of Bcl-2, DNA damage can occur, but not lead to apoptotic death. This dissociation between DNA damage and death in the presence of high Bcl-2 is the likely explanation for the resistance of certain tumors to chemotherapy and radiation treatment (Reed, 1994; Reed *et al.*, 1996). Similarly, in Alzheimer's disease, DNA fragmentation and upregulated Bcl-2 coexist in neurons without neurofibrillary tangles, suggesting that Bcl-2 may rescue cells at risk due to DNA fragmentation occurring as a result of the disease (Satou *et al.*, 1995; Su *et al.*, 1996; 1997). Indeed, it has been argued that DNA fragmentation in association with increased Bcl-2 may indicate an ongoing and potentially reversible process involving DNA repair (Cotman and Su, 1996). Since the odor-restricted olfactory bulb is the only other system in which both increased DNA damage and increased *bcl-2* expression have been reported, a similar interaction may be occurring here. In our experiments, the entire nucleus of most TUNEL(+) bulb cells was labeled uniformly, as compared to the nuclear marginalization and condensation of TUNEL positivity that is ususally associated with apoptotic

cell death. This finding may indicate early, and potentially reversible stages of DNA damage.

1.9. Translation of the bcl-2 Gene Family is a Regulated Process

Bcl-2 also can be regulated on the translational level, which means that the production of the protein also can be increased or decreased independently of gene expression. Evidence of translational control is seen in a variety of circumstances in which there are high levels of gene expression observed without correspondingly high protein levels (Kondo *et al.*, 1992; Chleq-Deschamps *et al.*, 1993). The sequences responsible for translational block have been identified (Harigai *et al.*, 1996). This translational regulation may be important in the bulb, as certain cell types in normal bulbs appear to contain high levels of *bcl-2* mRNA without showing appreciable immunostaining for the protein.

1.10. *bcl-2* Gene and Protein Expression are not Always Correlated in the Bulb

We have used commercially available antibodies to Bcl-2 to label cells expressing this protein at various ages in bulbs of nonodor-restricted rats (Najbauer, Okatani and Leon, unpublished observations). Interestingly, the distribution of immunoreactivity at P15 differed from the distribution of mRNA at the same age. Whereas *in situ* hybridization showed labeling of cell bodies in the glomerular layer as well as in the mitral cell and external plexiform layers, no appreciable immunostaining could be seen in cell bodies of the glomerular layer, despite good staining of mitral cells, tufted cells in the external plexiform layer, and what appear to be large cell types in the granule cell layer. Therefore, the translation of Bcl-2 may be blocked in juxtaglomerular cells during normal development, a situation similar to that reported in several blood cell lines (Kondo *et al.*, 1992; Chleq-Deschamps *et al.*, 1993). It seems possible that changes in neuronal activity or state could remove this translational block. That is, the protein may be expressed only when the cells are placed at increased risk during odor restriction or subjected to increased neuronal activity. Although it also is possible that the protein is being expressed at very low levels in their cell bodies, or in the processes of the neurons, rather than in their cell bodies, we have seen evidence for neither of these possibilities.

1.11. The Effects of Early Odor Restriction on Bulb Cell Death May Provide Novel Insights into the Regulation of Neuronal Survival and Death

Recent research has richly enhanced our understanding of the importance of neuronal death in neuropathology by using target ablation, axotomy, and ischemia to induce death (Oppenheim, 1991; Raff, 1992; Johnson and Deckwerth, 1993; Bredesen, 1995; Burek and Oppenheim, 1996). While these studies have greatly increased our understanding of neuronal death, they have tended to focus on traumatic insults to the nervous system, rather than studying the influences that less disruptive changes in neural activity have on cell survival (Oppenheim, 1991; Burek and Oppenheim, 1996). The clear effects on cell death by simple restriction of olfactory stimulation may make the olfactory system a particularly interesting system to study in this regard.

2. INCREASED EARLY OLFACTORY EXPERIENCE

2.1. Odor Preference Learning Increases Glomerular 2-DG Uptake

Rat pups develop preferences both for odors they experience in the nest and for odors that are paired with stimulation provided by the mother, e.g., tactile stimulation or the taste of milk. To determine whether these preferences are correlated with changed activity within the brain, pups were trained from postnatal days (PND) 1–18 to prefer peppermint odor by pairing it with tactile stimulation. They then were injected on PND 19 with [^{14}C]2-DG and exposed to the learned odor, as were pups that were trained using clean air. Exposure to peppermint odor resulted in focal accumulation of 2-DG in glomeruli located in the lateral part of the main olfactory bulb (Coopersmith and Leon, 1984). We have obtained similar results using [^{3}H]2-DG, which has a higher spatial resolution than [^{14}C]2-DG, and which shows that the uptake occurs principally in the glomerular neuropil (Woo and Leon, unpublished; see also Benson *et al.*, 1985). Trained animals accumulated more 2-DG in the peppermint-responsive lateral glomeruli than did controls (Coopersmith and Leon, 1984; Sullivan and Leon, 1986). The enhancement of 2-DG uptake following odor preference training is not due to increased respiration (Sullivan *et al.*, 1988), has a sensitive period for development (Woo and Leon, 1987; Sullivan and Wilson, 1995), lasts into adulthood (Coopersmith and Leon, 1986), and can be induced in response to the mother's natural odor (Sullivan, *et al.*, 1990).

2.2. The Enhanced Glomerular 2-DG Uptake Shows Odor and Regional Specificity

Uptake of 2-DG also increased in animals trained to prefer the pure odorant cyclohexanone, which induces 2-DG foci in the dorsomedial bulb (Coopersmith *et al.*, 1986). When rats were trained using cyclohexanone, 2-DG uptake increased in these dorsomedial glomeruli, but not in the lateral glomeruli activated by peppermint odor. Similarly, when rats were trained using peppermint odor, uptake did not increase in the dorsomedial glomeruli that respond to cyclohexanone. We also showed spatial specificity of changed mitral cell responses to different trained odors (Wilson and Leon, 1988).

2.3. The Learning-Dependent Enhanced 2-DG Uptake Shows Both Spatial Specificity and Effects Over the Entire Glomerular Layer

In order to explore further the spatial distribution of changes in metabolic activity, we developed a method to map activity across standardized regions of the glomerular layer in different animals (Johnson and Leon, 1996). When animals exposed to peppermint were compared to those exposed to air, fields of increased 2-DG uptake were detected in the lateral glomerular layer. The averaged pattern of uptake in response to peppermint odor was very similar in control and trained animals. The pattern included four discrete fields of higher uptake in the lateral glomerular layer, each field being separated from the others by intervening regions of lower uptake. The effect of learning became evident when the averaged array of the control animals were subtracted from that of the trained animals. The two groups differed the most in three of the four lateral fields of uptake, which emerged as distinct fields in the difference map. Uptake over the entire analyzed glomerular layer also was increased in the trained rats.

The maps in this study used the end of the lateral glomerular layer as the posterior limit. In subsequent experiments, we have analyzed the medial glomerular layer between this former boundary and the end of the medial glomerular layer. Five apparently discrete fields of higher uptake were identified in this posteromedial section of the bulb, and were similar in location in control and trained animals. Following training, the uptake in the four ventromedial fields of uptake increased to such a degree that these fields emerged as distinct fields in a difference map. One field did not increase in uptake to this extent.

2.4. Immediate-Early Genes Show Odor-Dependent Accumulation in the Glomerular Layer

To be able to observe the activity of the glomerular-layer neurons in addition to the activity within glomerular neuropil, we found that odor exposure causes c-*fos* mRNA accumulation in cells surrounding individual glomeruli (Guthrie *et al.*, 1993). The glomeruli labeled by *in situ* hybridization for c-*fos* mRNA correspond to the same glomeruli that take up 2-DG in these animals. Odorants that differ greatly in structure produce different patterns of glomerular c-*fos* mRNA signals (Guthrie *et al.*, 1993). Odor-dependent Fos-like and Egr-1-like immunoreactivity are present in cells surrounding glomeruli with the 2-DG uptake foci (Johnson *et al.*, 1995).

Fos and Egr-1-like immunopositive cells were counted around glomeruli showing 2-DG uptake in response to peppermint odor in trained and control animals (Johnson *et al.*, 1995). The number of periglomerular cells immunopositive for Fos-like antigens about doubled in trained animals, while the number of Egr-1 immunoreactive cells did not change. These data suggest that the trained odor initiates an increased response along a specific pathway in the olfactory bulb cells of trained pups.

2.5. Early Olfactory Preference Training Increases Olfactory Bulb Cell Number

One possibility for increased number of Fos-immunoreactive cells that are observed in response to a trained odor is that there is an increased number of neurons present in those areas as consequence of early olfactory preference training. In fact, morphometric analysis of the foci of enhanced 2-DG uptake reveals a 30% increase in width of the glomerular layer in odor-trained pups compared to controls (Woo *et al.*, 1987). The change in width is due to both an increase in the area occupied by glomerular neuropil and an increase in the area occupied by cell bodies. Furthermore, we found that trained pups have a 19% increase in the number of cells bordering these enlarged glomeruli in enhanced-uptake foci relative to controls (Woo and Leon, 1991). The periglomerular cells constitute the vast majority of this increase in cell number. Regions outside the 2-DG foci for the trained odor did not differ between trained and control groups. While the numbers of cells in a focus increased, the cell density did not, contributing to the increase in glomerular layer width observed in these regions. Since the periglomerular cells send dendritic and axonal processes into the glomeruli, the increase in periglomerular cell population could also contribute to the increased size of the glomeruli in trained pups. While the increase in cell number could have contributed to the increase in Fos-immunoreactive cells activated by a trained odor, the magnitude of that response indicates that there are both more cells and more cells responding to the trained odor.

2.6. Specificity of Cell Type for Changes in Survival

We found that there were no changes in the number or density of astrocytes as a consequence of early olfactory preference training, as visualized with glial fibrillary acidic protein immunohistochemistry (Matsutani and Leon, 1993). On the other hand, the density of immunoreactive processes was higher in glomeruli responsive to an odor for which pups had developed a preference. The increase in process density in trained pups was specific to the 2-DG focal responsive regions of the bulb. While there is an increase in the number of periglomerular cells that accompanies the increase in Fos-immunoreactive cells in trained pups, the decrease in Fos-immunoreactive granule cells in response to the trained odor is not accompanied by a change in the number of granule cells (McCollum, Woo and Leon, 1997; Woo, Oshita and Leon, 1996). The size of the granule cell somas did not change as a function of early experience. Finally, neither the size nor number of mitral cells changed following early olfactory preference training.

2.7. Functional Consequences of Changes in Cell Number

A functional change in the output signal of the bulb to learned odors can be related to the increased number of periglomerular cells we have observed. Periglomerular cells are inhibitory, and send axonal projections to mitral and tufted cell dendrites in adjacent glomeruli. Therefore one might expect that responses to olfactory stimuli emanating from that glomerular region might be more likely to be suppressed than activation of other glomerular regions or regions in pups with no olfactory preference conditioning. Indeed, single-unit recordings of mitral cells associated with the high 2-DG uptake foci in odor-trained pups reveal a decrease in excitatory and an increase in inhibitory mitral cell responses during peppermint odor presentation (Wilson and Leon, 1987). In addition, this suppression of activity is not observed in these same mitral cells during presentation of a novel odor (Wilson *et al.*, 1985), nor is it present in single-unit recordings from areas not typically associated with peppermint odor during the presentation of that odor (Wilson and Leon, 1988). This suppression of mitral cell responses also is not seen in pups trained with exposure to odor alone, tactile stimulation alone, or in naive controls (Wilson *et al.*, 1985; 1987; 1988). This change in responsiveness to the trained odor may well be the means by which the brain is alerted to this important odor.

2.8. Exposure to a Variety of Odors in Early Life Increases Cell Survival in the Bulb

While exposing young rats to a specific odor in the presence of a reinforcing stimulus activates the bulb in ways that alter the number of cells in the bulb, exposing young rats to an unusually wide range of odors in early life also increases the number of olfactory bulb cells. Specifically, when a variety of odors are introduced into the home cage from P1–21, there is an increase in the number of mitral and granule cells observed at P31 (Rosselli-Austin and Williams, 1990).

3. CONCLUSION

What mechanism underlies an increase in cell number consequent to an increase in early olfactory experience? It is possible that more neurons are produced in response to

the training, or that more neurons migrate into the area of the glomerular layer that is activated, or that the training reduces cell death in that area. If the latter mechanism is involved in this process, then it may be the case that the neuronal activity associated with early olfactory learning initiates a cascade of events that result in an increase in Bcl-2 within the periglomerular cells of the bulb. The Bcl-2 may then decrease the probability of an early death for this population of neurons and thereby increase their number following early olfactory preference training. This scenario is one that we will be studying in the coming years.

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6

TRANSNEURONAL SIGNALS FOR AFFERENT REGULATION IN THE CHICK AUDITORY SYSTEM

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1. INTRODUCTION

1.1. Activity and Development

Previous chapters in this volume have made it abundantly clear that the effects of early sensory deprivation are robust. In fact, an important role of sensory experience has been implicated in the development of every sensory system thus far examined, (this volume, also see for example, Levi-Montalcini, 1949; Wiesel and Hubel, 1963a,b; 1965; Peusner and Morest, 1977; Webster and Webster, 1977; Mistretta and Bradley, 1978; Stryker and Harris, 1986; Frazier and Brunjes, 1988; Rubel *et al.*, 1990). This role of early experience has been documented using a variety of procedures for manipulating sensory input. One common approach is to examine changes in neural development after raising the animal without exposure to the sensory stimulus, such as by rearing an animal in the dark or raising them in an "impoverished" environment. Alternatively, peripheral sensory structures could be modified to prevent the detection of the sensory stimulus. In the previous chapter by Johnson and Leon, for example, the naris was occluded to limit exposure to olfactory cues. A more extreme manipulation is to totally abolish sensory processing in a given system by damaging the sensory receptors or primary sensory nerves, as seen in the earlier chapter by Parks in which the otocyst was removed. From a psychological point of view, these various manipulations would certainly be different in terms of how they change the organism's perceptual experience. From the perspective of the developing neuron, however, the important aspect of these manipulations is how they change the activity of the sensory afferents, not the perceptual outcome. In other words, regardless of the exact type of manipulation, any effects of these manipulations can be attributed to the fact that the manipulation produced a change in the rate or

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pattern of activity in the sensory nerves. Somehow, these changes in afferent activity regulate the development of CNS neurons.

This regulatory influence of neural activity appears to be a common property in the development of various sensory circuits in the brain. In addition, afferent activity regulates the structure and function of muscle fibers (Guth, 1968; McArdle, 1983; Lømo and Gundersen, 1988), and is important for maintaining neurons in the autonomic nervous system (Maderdrut *et al.*, 1988). Thus, the influence of activity on development appears to be a general property of the developing organism. Without the typical sensory afferent input, neurons will be reduced in size, develop atypical innervation patterns, or even die.

1.2. Model System

Despite the seemingly ubiquitous influence of afferent activity on neuronal structure, function, and viability, relatively little is known of the cellular mechanisms that underlie these effects. Many of the chapters in this volume attempt to understand the cellular underpinnings of such early influences. This chapter will focus on work directed at understanding the cellular events underlying the afferent regulation of cells in the brain stem auditory system of the chick. In this system, as is the case in many other sensory systems, elimination of afferent activity in young animals results in the death and atrophy of postsynaptic neurons.

The brain stem auditory system of the chick is a relatively simple system and much is known of its normal structure, function and development (Rubel and Parks, 1988). This system has been described in the previous chapter by Parks and is displayed again in Fig. 1. Briefly, the auditory nerve enters the brain stem and bifurcates, sending one branch to nucleus magnocellularis (NM) and the other to nucleus angularis. NM is homologous to the mammalian anteroventral cochlear nucleus. Neurons in NM receive their input from the auditory nerve in the form of a large, secure synapse called an endbulb of Held. Each NM neuron projects bilaterally to nucleus laminaris (NL, similar to the mammalian medial superior olive). Neurons in NL form a monolayer of cells with symmetric dorsal and ventral dendrites. Input from the ipsilateral NM synapses on the dorsal dendrites of NL neurons, while input from the contralateral NM synapses on the ventral dendrites. Neurons in NM and NL are also contacted by small punctate terminals containing the inhibitory neurotransmitter, GABA, from descending connections from the superior olive (Lachica *et al.*, 1994) and from GABAergic neurons scattered between and around NM and NL (Code *et al.*, 1989; von Bartheld *et al.*, 1989).

1.3. Afferent Influences

As seen in the chapter by Parks, the brain stem auditory system of the chick has been used successfully to examine the effects of afferent activity on CNS development. Since the auditory nerve projects only to the ipsilateral NM, unilateral manipulations of auditory nerve activity affect afferent input to ipsilateral NM, leaving input to the contralateral NM intact and normal. The most extreme manipulation, deafness, results in an immediate cessation of activity in the ipsilateral NM (Born *et al.*, 1991). Thus, a common approach has been to remove one cochlea (basilar papilla), or the embryonic otocyst, and examine differences between the deafferented and control NM in the same animal.

Cochlea removal has rapid and dramatic effects on NM neurons. In young birds,

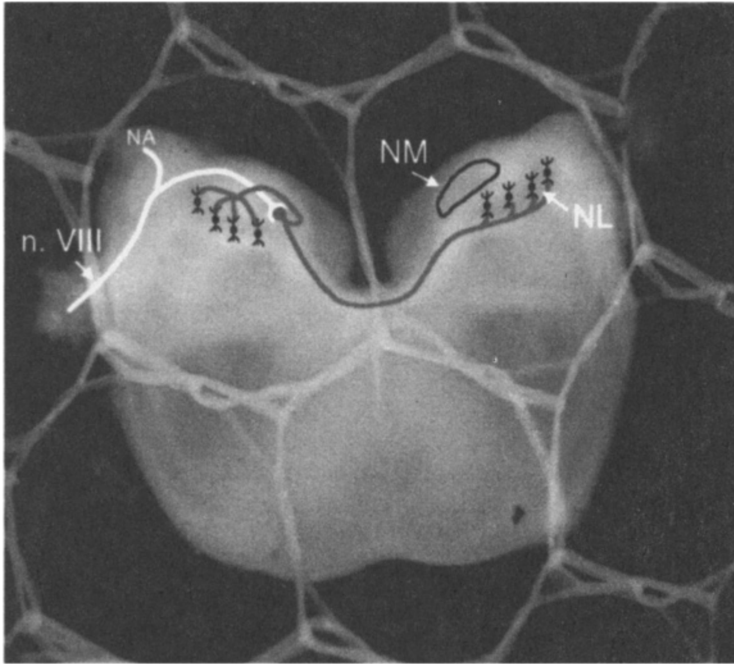

Figure 1. Coronal slice of the chick brain stem with a schematic overlay of the auditory system. The photomicrograph is of a 300 μm slice as it appears when it is maintained *in vitro*. The nylon mesh on top of the slice is used to hold it in place as media flows through the chamber. As shown schematically, the auditory nerve (n. VIII, white line) enters the brain stem and projects to the cochlear nuclei, nucleus angularis (NA) and nucleus magnocellularis (NM). Each NM neuron (gray) sends projections bilaterally to neurons in nucleus laminaris (NL).

some neurons in the ipsilateral NM die and the rest shrink in size within two days after cochlea removal (Born and Rubel, 1985). These effects show an age-dependence; pronounced effects are observed when the cochlea is removed in embryonic or posthatch chicks up to 6 weeks of age, but little or no effect is observed in adult (1 year old) birds. Age-dependence is a relatively common feature in the regulation of neuronal morphology by sensory experience. The so-called "sensitive" or "critical" period over which manipulations of sensory experience has the most profound effect is often highlighted as the reason for enriching a child's early sensory environment. Why adult neurons are not as sensitive to manipulations of the sensory environment is not understood. Recent work by Durham and colleagues show that the age dependence of these effects are not observed in all strains of chickens (Edmonds *et al.*, 1999). It appears that strains bred selectively for egg laying are sensitive to deafferentation throughout their lives, while "broiler" strains that are bred to provide meat show an age dependence. These are intriguing results which may lead to a fuller understanding of the biological nature of this sensitive period. One of the long range goals of the work presented in this chapter is also to understand the biological underpinnings of the enhanced sensitivity of young organisms. An important first step is to understand the cascade of events involved in the transneuronal regulation of young sensory neurons. Then comparative studies using young and old birds can focus on key elements of this cascade to determine what aspects of the signaling cascade have changed with age.

The immediate goal of the work to be presented in this chapter is to understand how activity from the auditory nerve assists in the survival and health of postsynaptic neurons in the avian cochlear nucleus. The effects of deafferentation that occur over the time span of days to weeks, such as cell death, are of interest because of long lasting changes in function that would be associated with such profound alterations. However, it is difficult to determine the identity of the transneuronal signals responsible for effects that take so long to detect. In the case of cochlea removal, the activity of the auditory nerve afferents to NM stops virtually immediately (Born *et al.*, 1991). Thus, it is likely that changes in activity-dependent signals occur instantaneously and the processes leading toward cell death and atrophy may begin relatively rapidly. To investigate the nature of the moment-to-moment signaling that may be necessary for maintaining healthy neurons, a more rapid assay is desirable. Fortunately, rapid changes in NM after cochlea removal have been observed using various indices of metabolic activity. These include a decrease in overall protein synthesis, decreased concentrations of specific structural proteins, and a biphasic change in the activity of oxidative enzymes. The time course of these various effects have been reviewed previously (see Rubel and Parks, 1988; Rubel *et al.*, 1990).

The changes in protein synthesis observed after cochlea removal are particularly interesting. This has been evaluated by injecting birds with a radiolabeled amino acid at various times after cochlea removal. Cells take up the labeled amino acid and incorporate it into proteins. When the tissue is subsequently processed using autoradiography, differences in the relative amounts of protein synthesis can be detected between NM neurons on the deafferented side compared to those on the normal side of the brain. Protein synthesis is reduced in deafferented NM neurons as early as 0.5 hr after cochlea removal (Steward and Rubel, 1985). By 6 hr, autoradiographic analysis of protein synthesis can segregate neurons in the deafferented NM into two populations: one that stops making protein and one that shows a reduced level of synthesis compared to the control side of the brain. It appears that the neurons that stop making protein are those destined to degenerate over the next few days, while those with reduced synthesis will survive but will shrink in size. Thus, protein synthesis changes occur rapidly and appear to be a good predictor of the long-term fate (life or death) of the neurons.

The reduction in protein synthesis could simply be a reflection of reduced RNA synthesis (Garden *et al.*, 1995a), but it could also result from a reduction in synthetic activity of the ribosomes. There is a notable relationship between the reduction of protein synthesis and ribosomal structural integrity. For example, electronmicroscopic analyses have shown that the cells that stop making protein 6 hr after deafferentation also show a loss of ribosomes along the rough endoplasmic reticulum and a dissociation of polyribosomes (Rubel *et al.*, 1991). Thus, it appears that the loss of protein synthesis may be attributable to the breakdown of the ribosomes.

Ribosomal changes after cochlea removal have also been observed by immunolabeling using an antibody (called Y10B) which recognizes ribosomal RNA (Lerner *et al.*, 1981). This assay is used for many of the experiments reviewed in this chapter. Reductions in Y10B labeling are apparent well before any evidence of ribosomal destruction is observed by EM analysis (Rubel *et al.*, 1991; Garden *et al.*, 1994). Therefore, the early reduction in Y10B labeling probably reflects a change in the epitope on the ribosomal RNA and not the destruction of ribosomes, per se. Additionally, the deafferentation-induced differences in Y10B antigenicity are not observed if the tissue is treated with a protease prior to labeling (Garden *et al.*, 1995b). This suggests that the earliest changes in labeling emerge because some protein has bound to the ribosome and blocked the

epitope or caused a change the conformation of the epitope. Regardless of the cause for the change in antigenicity, the change observed by Y10B immunolabeling appears to be functionally significant. The time course of changes in ribosomal function (protein synthesis) parallels the time course of change in Y10B antigenicity, and the later-occurring ribosomal dissociation can also be seen by the loss of Y10B staining (Garden *et al.*, 1994; Hyson and Rubel, 1995). Thus, decreased Y10B binding appears to correspond to a lower capacity of the ribosome to synthesize protein.

2. THE BIOLOGY OF EARLY AFFERENT INFLUENCES

2.1. Afferent Activity

As noted earlier, from the perspective of the CNS neuron, changes in experience are defined as changes in the afferent activity detected by that particular neuron. Many studies examining the effects of early experiences suffer from the lack of knowledge of how afferent activity has changed. Many investigators rationally assume that activity has changed as a result of the experimental manipulation, but frequently there is no specific knowledge of how the activity is different. An experiment performed by Tucci *et al.* (1987; Tucci and Rubel, 1985) provided some surprising results that perhaps exemplify the danger of making such assumptions. Measurements of activity in NM have shown that after the extreme manipulation of unilateral deafening, afferent excitatory activity has completely stopped. Tucci *et al.* asked whether a less extreme manipulation of sensory experience would also influence the viability of NM neurons. Conductive hearing losses were produced by rupturing the tympanic membrane or removing the bird's single ossicle, the columella. Surprisingly, although these manipulations produced large shifts in the auditory evoked potential thresholds, there was no effect on either cell size or cell number. Reduced cell size and number were only observed if the cochlea was damage by puncturing the oval window. Electrophysiological recordings from NM following these manipulations provided a logical interpretation of these results. Although the conductive hearing loss did increase thresholds for driven activity, they did not affect the abundant spontaneous activity that exists if the cochlea is not damaged. Apparently, the high level of spontaneous activity is sufficient for maintaining healthy neurons, and the cessation of spontaneous activity causes NM cells to degenerate and atrophy.

An alternative interpretation for the effects of cochlea damage is that the transneuronal changes do not result from of a loss of afferent activity, but rather, results from degenerative changes in the auditory nerve. In studies of deafferentation where the receptors are removed, as is the case with cochlea removal, there is likely to be damage to the distal processes of the sensory afferents. To demonstrate that the transneuronal changes are produced by changes in activity, not damage, Born and Rubel (1988) blocked the auditory nerve activity without causing permanent damage to the nerve by injecting tetrodotoxin (TTX) into the perilymph of the inner ear of young chicks. The TTX blocks the generation of action potentials in the auditory nerve but does not destroy the fibers. TTX treatment produced the same changes in protein synthesis, cell size and cell number as were observed in the experiments in which the cochlea was removed (Born and Rubel, 1988). The lack of permanent damage is evidenced by the fact that some of these changes are reversible if the TTX is administered only for a short while and activity is allowed to return when the drug wears off. Similar effects of blocking auditory nerve activity occurs in the mammalian brain stem auditory system (Pasic and Rubel, 1989). As in the chick,

after 24–48 hr of activity blockade, spherical cells in the ipsilateral cochlear nucleus decreased in size. The effects of TTX were identical to those following cochlea removal. Thus, alterations in the *activity* of sensory afferents account for the transneuronal regulation of cellular metabolism and integrity. The results of the TTX studies and the study by Tucci (Tucci and Rubel, 1985), reviewed above, demonstrate the importance of so-called "spontaneous" activity of the sensory nerve fibers. A variety of other studies have also shown that spontaneous action potentials are important determinants in guiding neural development (e.g., Fields and Nelson, 1992; Spitzer *et al.*, 1994; Katz and Shatz, 1996). Although intrinsically driven action potentials may not be the type of thing one immediately thinks about when discussing the role of sensory "experience", it is important to keep in mind that the postsynaptic neuron does not "know" whether the inputs it receives are spontaneously or sensory driven. Rather, the postsynaptic neuron only "knows" that there is a transmitter substance binding to its receptor. Thus, from the point of view of the postsynaptic cell, elimination of spontaneous activity is simply an extreme reduction in its "experience".

What is it about afferent activity that influences the survival of the NM neuron? We see that afferent activity is crucial in a variety of sensory systems, but it is not clear what sort of trophic support it provides to the postsynaptic neuron. The goal of the research presented here is to understand the biology of this early influence, rather than "merely" identifying the phenomenology. A first step towards this goal is to understand the signals used for communication between neurons. These transneuronal signals serve two essential functions. First, chemical signals from the presynaptic terminal regulate the *electrical activity* of the postsynaptic neuron. This chemical synaptic transmission is the most common mechanism of relaying neural information and, thus, is crucial for the neural coding and processing of acoustic information. A second, and sometimes neglected function of transneuronal signals is their role in regulating the *metabolism* and consequently, the health of the postsynaptic neuron (as documented above). This form of transneuronal communication is especially important in developing animals, and it is also observed in various forms of "neuronal plasticity" resulting from changes in neural activity (e.g., learning, disease states, etc.). Thus, a major objective of the work describe in this chapter is to identify transneuronal signals involved in regulation of NM neurons.

2.2. *In Vitro* Analysis

The signals involved in afferent regulation of neuronal structure and metabolism have been investigated by using a slice preparation of the brain stem auditory system to mimic the *in vivo* situation of unilateral cochlea removal. The brain stem slice contains the auditory nerve and NM on both sides. Obviously, the cochlea is removed on both sides of the slice, so NM is deprived of afferent input on both sides. To mimic the *in vivo* condition of unilateral cochlea removal, the auditory nerve on one side is electrically stimulated. Since the innervation of the auditory nerve to NM is strictly unilateral, the NM on the stimulated side of the slice receives afferent input (analogous to the intact cochlea side), while NM on the unstimulated side would be deprived of afferent input (analogous to the cochlea removal condition). One advantage of this preparation over the *in vivo* situation is the ability to perform pharmacological manipulations which can help identify the signaling cascade involved in the transneuronal regulation of neuronal structure and metabolism.

A variety of bioassays could be used to evaluate the effects of afferent activity. The

major restriction for *in vitro* studies is that the assay measures a change that occurs relatively rapidly, since the brain slice will eventually die. The assays we have used for the *in vitro* studies have examined the integrity of the ribosomes in the NM neuron. The change in ribosomal integrity is an optimal assay because it is observed within an hour after deafferentation, allowing for an *in vitro* analysis of these changes in an acute slice preparation. As noted above, the two most promising assays for ribosomal integrity have been changes in protein synthesis and changes in immunoreactivity using the Y10B antibody (which recognizes ribosomal RNA). These changes are observed after only one hour of differential afferent activity. In additional to the practicality of using a rapid change for *in vitro* experiments, these assays were also chosen because changes in the ribosomes appear to be particularly important, as they appear to be among the first in a progression of events resulting in the eventual atrophy or death of the neurons (Rubel *et al.*, 1990; Rubel *et al.*, 1991; Garden *et al.*, 1994). In NM, the early changes in ribosomal structure and function appear to be early indicators of which cells will die and which will atrophy, but survive.

Unilateral stimulation of the auditory nerve *in vitro* produces changes in ribosomal integrity that are similar to those observed after unilateral cochlea removal *in vivo*. Within one hour, NM neurons on the stimulated side of the slice show greater protein synthesis than those located on the opposite side of the same slice (Hyson and Rubel, 1989). Similarly, stimulated NM neurons show greater antigenicity for the ribosomal antibody, Y10B (Hyson and Rubel, 1995). An example of these effects is shown in Fig. 2.

2.3. Activity or Synaptic Signals?

While both *in vivo* and *in vitro* evidence have pointed to a role of afferent activity in regulating ribosomal integrity, it was still unclear *how* afferent activity produced these effects. The analysis of this question began by determining whether activity of the postsynaptic NM neuron is sufficient to begin the cascade of events necessary for maintaining ribosomal integrity. Electrical stimulation of the auditory nerve leads to action potentials and greater protein synthesis in the postsynaptic NM neuron. Would action potentials in the NM neuron alone also result in greater ribosomal function or does this metabolic regulation require the action of some substance released from the auditory

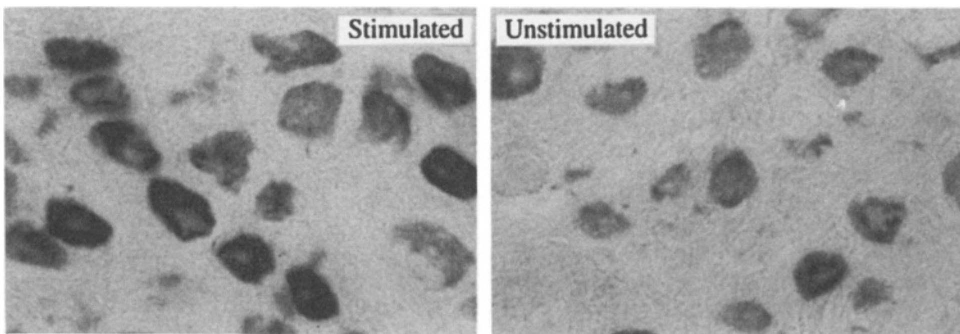

Figure 2. Example of the activity-dependent difference in staining when using the ribosome-specific Y10B antibody. In this example, the auditory nerve on one side of the slice was stimulated at a rate of 10 Hz for one hour. This short period of stimulation is sufficient to cause reliable differences in labeling. As can be seen in this example, stimulated neurons are more darkly stained than unstimulated neurons on the opposite side of the same tissue section. Quantitative data shown in Fig. 4.

nerve? This question was addressed in the slice preparation by electrically driving action potentials in the NM neuron without first activating the auditory nerve. NM neurons were activated by antidromically stimulating their axons where they crossed midline. This produced action potentials that could be recorded at the soma, but did not involve the various signaling cascades initiated by synaptic stimulation. For both protein synthesis (Hyson and Rubel, 1989) and Y10B immunolabeling (Hyson and Rubel, 1995), antidromic stimulation did not produce greater labeling on the stimulated side of the slice. In fact, for both assays, the labeling was reliably lower on the stimulated side compared to the unstimulated side of the same slice. Electrophysiological experiments showed that the different effects of orthodromic and antidromic stimulation were not simply due to a differential production of action potentials recorded at the soma. Thus, action potentials, per se, are not the major signal responsible for the greater ribosomal integrity observed when the neuron is driven orthodromically.

The data using antidromic stimulation of NM neurons are quite different than what would be expected based on studies at the neuromuscular junction. Deafferentation of muscle fibers produces a host of changes in the postsynaptic cell. However, electrical stimulation of the muscle can attenuate or reverse many of these effects (Lømo and Rosenthal, 1972; Lømo and Westgaard, 1975; Lømo and Gundersen, 1988). In NM, on the other hand, electrical stimulation of the postsynaptic cell, if anything, exaggerates the detrimental effects of deafferentation.

The antidromic stimulation experiments suggest that the influence of auditory nerve activity on ribosomal integrity in the NM neuron is not merely due to some factor related to the production of action potentials in the postsynaptic cell, but rather, that something must be released from the auditory nerve terminal in order for this form of transneuronal regulation to occur. The hypothesis that synaptic release is necessary was also supported by experiments in which synaptic release was prevented by maintaining the slice in a medium having low Ca^{2+} and high Mg^{2+} concentrations. Neither differential protein synthesis (Hyson and Rubel, 1989) nor Y10B labeling (Hyson and Rubel, 1995) was observed when the auditory nerve was stimulated in low Ca^{2+} medium. The most plausible explanation for this matrix of data is that some trophic factor, which is released from active auditory nerve terminals, influences the protein synthesis machinery in the postsynaptic cell. The next step, then, is to identify the trophic substance released from the auditory nerve and determine the cascade of cellular events initiated by this signal.

2.4. Potential Synaptic Signals

The fact that action potentials are not the critical factor for maintaining the postsynaptic cell does not preclude the possibility that neurotransmitters have a trophic influence on the NM neurons. Any of the events in the cascade from receptor activation to the generation of the action potential could be important for the maintenance of the neuron. These events would not necessarily be activated by antidromic stimulation of the neuron. Additionally, a given neurotransmitter can act on a variety of receptor types and metabolic regulation may result from the cascade of events after activation of a different receptor than that used for generating the excitatory postsynaptic potentials leading to action potentials.

Based on electrophysiological studies using receptor agonists and antagonists, the neurotransmitter from the auditory nerve to NM is an excitatory amino acid (Nemeth *et al.*, 1983; Jackson *et al.*, 1985; Martin, 1985; Zhou and Parks, 1992b). Although there

are a variety of excitatory amino acid receptors, this work has shown that a non-NMDA ionotropic type of receptor is responsible for the fast excitatory communication from the auditory nerve to the NM neuron. The NMDA type of glutamate receptors have also been found in NM, but their contribution to the synaptic current appears to wane with increasing age. By the time the chick has hatched, most of the excitatory current is attributable to non-NMDA receptors.

To determine if glutamate is also involved in the regulation of neuronal metabolism we made use of the pharmacological profile of NM glutamate receptors (identified by Parks and others). The logic was that if the neurotransmitter is serving a trophic role for NM neurons, then the effects of stimulation should be reduced by pharmacological blockade of the neurotransmitter receptor. The auditory nerve was unilaterally stimulated in slices from young chicks (orthodromic stimulation), and groups of slices were treated with different glutamate receptor antagonists added to the circulating medium. The drug APV (50 μM) was added to block NMDA receptors and CNQX (20 μM) was added to block the non-NMDA receptors. Electrophysiological studies using these antagonists confirmed what has previously been reported. First, the NMDA receptor antagonist produced no noticeable effect on the electrophysiological responses of NM neurons from these 1–2 week old chicks. The non-NMDA receptor antagonist, however, completely blocked synaptic transmission at this synapse. Samples of this effect on both field potential and intracellular recordings can be seen in Fig. 3. Average (16 traces) field potentials recorded from NM during stimulation of the auditory nerve are shown in Fig. 3A. For these studies a relatively large tipped micropipette, filled with slice media, was placed on NM. The form of the potentials take the typical double-dip appearance with the first downward deflection being the response attributable to the activity of the auditory nerve fibers and the second downward deflection being the response attributable to the postsynaptic activity of the NM neurons. This interpretation of the potentials is verified by the fact that 1) the second deflection, but not the first, is eliminated when synaptic release is prevented by maintaining the slice in a low Ca^{2+}, high Mg^{2+} medium (not shown) and 2) the non-NMDA glutamate receptor antagonist CNQX prevents the second deflection but not the first. Figure 3B shows single traces recorded intracellularly from an NM neuron following auditory nerve stimulation. In normal media, NM neurons can be driven to produce an action potential when the auditory nerve is stimulated. CNQX completely blocked the action potential and any underlying EPSPs in NM neurons.

The physiological data show that blockade of the non-NMDA glutamate receptors blocks synaptic transmission from the auditory nerve to NM. The next question is whether blockade of these receptors would also prevent the afferent regulation of ribosomes in NM neurons. To address this question, the auditory nerve was unilaterally stimulated for one hour in media containing glutamate receptor antagonists. After stimulation, the slice was fixed, and processed for Y10B immunoreactivity to evaluate ribosomal integrity. Surprisingly, these glutamate receptor antagonists had no noticeable effect on the afferent regulation of ribosomes in NM. Whether in normal media, media containing the non-NMDA receptor antagonist CNQX, media containing the NMDA receptor antagonist APV, or media containing both CNQX and APV, the NM neurons on the stimulated side of the brain showed greater labeling than neurons on the opposite side of the same slice.

The lack of effect of glutamate receptor antagonists on this assay of ribosomal integrity is interesting in light of the fact that CNQX completely blocks the synaptic drive of NM neurons under these conditions. Thus, there appears to be a dissociation in the

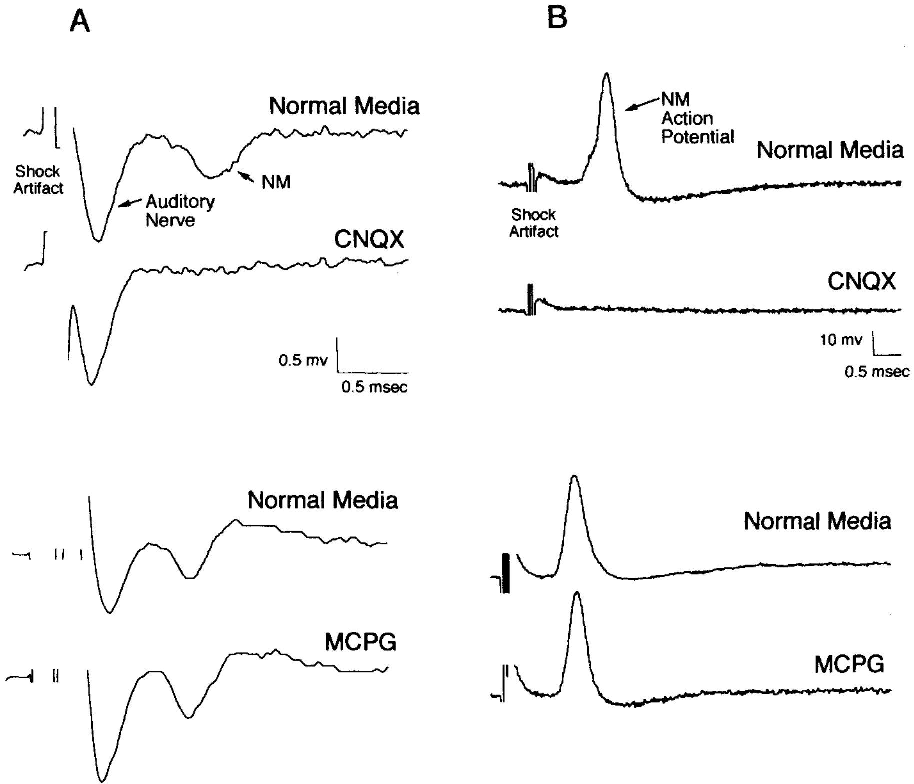

Figure 3. Synaptic drive in this system is mediated by non-NMDA glutamate receptors. Field potentials (A) recorded over nucleus magnocellularis (NM) typically show two downward deflections: one attributable to the activity of the auditory nerve fibers and one attributable to the postsynaptic activation of NM neurons. The non-NMDA glutamate receptor antagonist CNQX (20μM) blocks the postsynaptic potential. Intracellular recordings (B) further show that no excitatory postsynaptic potentials are evoked with the auditory nerve is stimulated when CNQX is in the medium. Field potentials and intracellular spikes appear to be unaffected by the metabotropic glutamate receptor antagonist MCPG.

signaling pathways by which afferent fibers control the electrical activity of the postsynaptic neuron and the signaling pathways by which afferents control the metabolic activities of the neuron.

At first glance, these data may appear to be at odds with Parks' (this volume; Solum *et al.*, 1997) recent work on cell death in chick embryos. In those studies, *in ovo* treatment with the glutamate antagonist CNQX prevented both naturally-occurring cell death in NL and deafferentation-induced cell death in NM. In our studies, the same pharmacological agent, CNQX, had no effect on afferent regulation of NM neurons. There are several possible reasons for this apparent discrepancy. As Parks points out, however, the effects of the *in ovo* treatment are observed even before functional synapse are made in this system. Consequently it is possible that this early treatment is having a systemic effect that aids the survival of neurons. Perhaps, for example, CNQX treatment upregulates the release of some systemic trophic agent that rescues cells. Based on the current state of our knowledge about the chick's auditory system, Parks' hypothesis makes sense. If CNQX was presented to the whole animal after the onset of cochlear function, then the drug should produce a functional deafferentation to both ears since glutamate receptors

at the distal processes of the auditory nerve would also be blocked bilaterally. Thus, one would have to reconcile how functional deafferentation using CNQX leads to enhanced cell survival, whereas physical deafferentation by otocyst removal leads to cell death. Since the brain slice is isolated in our studies, there obviously is no opportunity for upregulating any possible systemic trophic substances.

A second potential cause for the apparent discrepancy between the effects of embryonic CNQX treatment and the lack of effect observed *in vitro*, is that assays are different and the effects are measured at different points after deafferentation. The brain slice studies examined very early occurring activity-dependent events, whereas Parks *et al.* examined long-term effects. Perhaps CNQX does not alter the early effects of deafferentation, but does influence the long-term survival of these neurons. Finally, the discrepancy could also be attributable to age differences of the subjects. Parks *et al.* examined the effects of CNQX in embryonic chicks (otocyst removal at E3 with cell counts at E17), whereas the chicks used in the brain slice studies were 1 to 2 weeks posthatch. The late period of embryonic development appears to be one of rapid change in the auditory system of the chick. Importantly, Parks (this volume, Zhou and Parks, 1992a) has shown that the pharmacological profile of glutamate receptors in NM appears to change during this period. Thus, it is possible that there is a developmental change in influence of glutamate receptors on cell death. If this is the case, then activation of non-NMDA glutamate receptors must participate in the initiation of cell death in embryonic chicks, but later in development these receptors play no significant role in cell survival.

2.5. Metabotropic Glutamate Receptors

The previous data suggest that the signaling cascade responsible for the afferent regulation of ribosomal integrity is different than the signaling cascade regulating electrophysiological activity. What, then, is the signaling cascade responsible for the afferent regulation of ribosomal integrity? It is possible that active auditory nerve terminals corelease the neurotransmitter glutamate as well as some trophic substance which has a transneuronal metabolic influence. Alternatively, it is possible that the neurotransmitter glutamate is also responsible for the regulation of ribosomal integrity, but that this effect is mediated thorough the activation of metabotropic glutamate receptors (mGluRs), which would not have been blocked in the previous experiment using ionotropic antagonists. Activation of mGluRs influence several second messenger systems but they do not necessarily play a major role in the fast transmission of electrical signals. In other systems, activation of mGluRs has been shown to influence the protein synthesis capacity of neural tissue (Weiler and Greenough, 1993) and to protect some neurons from cell death (Bruno *et al.*, 1996). Perhaps mGluRs are also involved in the transneuronal regulation of the protein synthesis machinery in NM neurons. There are a variety of mGluRs (Conn and Pin, 1997) and previous studies using biochemical assays (Zirpel *et al.*, 1994) and Ca^{2+} imaging (Lachica *et al.*, 1995; Zirpel *et al.*, 1995b) have shown that mGluRs are present in NM of the chick.

To determine if mGluR activation is necessary for the afferent regulation of ribosomal integrity, NM neurons were unilaterally stimulated while blocking metabotropic glutamate receptors with the antagonist MCPG. Once again, the auditory nerve was stimulated for one hour *in vitro* and then processed using immunocytochemistry for the ribosome-specific Y10B antibody. Different groups of slices were stimulated either in normal media, or in media containing the metabotropic antagonist MCPG (either 0.1 or

0.25 mM). Electrophysiological studies documented that MCPG did not have any obvious effect on the electrophysiological responses of NM neurons produced by electrical stimulation of the auditory nerve. An example of this apparent lack of effect can be seen in Fig. 3. In contrast to the effects of CNQX, there was no consistent change in the amplitude of the postsynaptic field potential and it was clear that EPSPs and action potentials were still readily produced in the presence of the metabotropic antagonist. These results reconfirm that synaptic transmission is primarily governed by activation of non-NMDA ionotropic glutamate receptors.

The anatomical assay of ribosomal integrity, however, showed a quite different pattern of results. Once again, stimulation of the auditory nerve in normal media resulted in greater labeling of NM neurons on the stimulated side of the slice. However, when orthodromically stimulated in 0.1 mM MCPG, the difference between stimulated and unstimulated sides was reduced. In 0.25 mM MCPG, there was no reliable difference between sides and, if anything, the stimulated neurons were stained lighter than those on the unstimulated side of the same slice. Thus, it appears that in order for afferent activity to maintain ribosomal integrity in NM neurons, metabotropic glutamate receptors must be activated.

2.6. Summary and Quantification

The brain stem auditory system of the chick provides a good model for analyses of the effects of deafferentation because a unilateral manipulation allows one to compare experimental and control populations of neurons in the same tissue section. It is important for these studies to measure labeling on opposite sides of the same section because various processing variables (e.g., time in antibody, time in DAB, etc.) could influence the absolute labeling density. By measuring cells in the same section, one can directly compare labeling in stimulated and unstimulated NM neurons without these potential confounding variables. For these analyses, the video image of neurons viewed under a 40× objective was digitized and analyzed using NIH Image software. For each brain, the labeling density of approximately 40 individual NM neurons was measured on each side of the same tissue section. These gray scale measurements were then transformed to z-scores based on the mean and standard deviation of labeling measured on the unstimulated sided of the slice. This z-score transform allowed the data to be combined across slices for statistical and graphical purposes by normalizing for differences in the absolute level of staining between different slices. The group z-score data for all of the experiments described in this chapter are displayed in Fig. 4. Positive numbers using this measure mean that there was greater labeling on the stimulated side of the brain. An average z-score of 1, for example, indicates that the labeling density on the stimulated side was, on average, 1 standard deviation greater than the mean labeling density of cells on the unstimulated side of the slice.

When the auditory nerve was stimulated in normal media (group Ortho), NM neurons are more darkly labeled with the Y10B antibody on the stimulated side of the brain. As noted above, this difference in labeling was not observed when the NM neurons were stimulated antidromically (group Anti). In fact, antidromically driven NM neurons show reliably less labeling than unstimulated neurons on the opposite side of the same slice. Orthodromic stimulation in a medium containing low Ca^{2+} and high Mg^{2+} concentrations (group Low Ca^{2+}) resulted in no reliable difference between the two sides, presumably caused by the low Ca^{2+} media preventing synaptic release from the stimulated

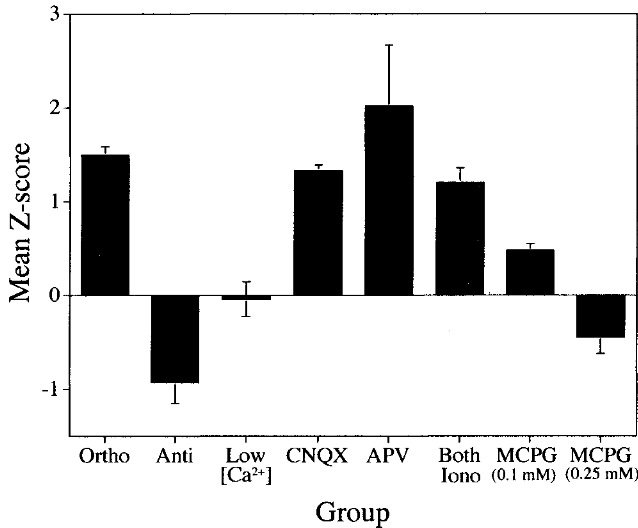

Figure 4. Mean difference in labeling for the various groups discussed in this chapter. The density of immunostaining was measured for cells on opposite sites of the same tissue section. The data from each slice were converted to z-scores based on mean and standard deviation of density measurements for the cells on the unstimulated side of that slice. Thus, z-scores greater than 0 mean that the immunostaining was darker on the stimulated side of the slice. All slices were stimulated unilaterally for 1 hr at a rate of 10 Hz. For group Anti, the axons of NM fibers on one side of the slice were stimulated to produce antidromic action potentials. For all other groups, NM was orthodromically stimulated by electrical stimulation of the auditory nerve. The key difference is that these groups were maintained and stimulated in different media. The media for each group differed as follows: Group Ortho, normal medium; Low Ca^{2+}, medium had low Ca^{2+} and high Mg^{2+} concentrations; CNQX, medium contained 20 μM of this non-NMDA glutamate receptor antagonist; APV, medium contained 50 μM of this NMDA glutamate receptor antagonist; Both Iono, medium contained both ionotropic glutamate receptor antagonists CNQX and APV; MCPG, medium contained either 0.1 or 0.25 mM of this metabotropic glutamate receptor antagonist. See text for interpretation of these results.

auditory nerve fibers. Orthodromic stimulation while blocking NMDA receptors, non-NMDA receptors, or combined blockade of both subtypes still resulted in a reliable difference in immunolabeling between the "stimulated" and unstimulated neurons (Groups CNQX, APV and Both Iono). Finally, orthodromic stimulation in the presence of the metabotropic glutamate receptor antagonist, MCPG, attenuated (MCPG 0.1 mM) or eliminated (MCPG 0.25 mM) the difference in labeling between the stimulated and unstimulated sides of the slice.

In summary, although CNQX blocked electrophysiological response of NM neurons driven by stimulating the auditory nerve, it did not prevent the afferent regulation of ribosomal integrity. In contrast, MCPG had no observable effect on the electrophysiological responses of NM neurons but prevented the afferent regulation of ribosomal integrity. Thus, it appears that glutamate serves at least two roles in the regulation of NM neurons, each of which is mediated by a separate receptor system. First, through its action at non-NMDA ionotropic receptors, glutamate is responsible for the fast excitatory synaptic responses in NM neurons that are needed for coding and relaying information about the bird's acoustic environment. Second, through the action at mGluRs, glutamate plays a trophic role that regulates the metabolic integrity of NM neurons.

One implication of these studies is that it is not the loss of activity per se, but rather, the loss of mGluR activation that leads to the metabolic consequences of deafferentation. In fact, activity in the form of action potentials in the postsynaptic neuron is not necessary for cell viability, but instead, could have detrimental consequences (e.g., group Anti, in Fig. 4). Detrimental effects of activity is commonly assumed to be the case in other areas of the brain, such as the hippocampus, where high levels of activity result in excitotoxicity (Sloviter *et al.*, 1996). It is intriguing that the data from the higher dose of mGluR antagonist resemble those in which cells were antidromically stimulated. In both cases, the stimulated cells show less immunolabeling than cells on the unstimulated side of the slice. Although the reduction in labeling was not statistically reliable in the MCPG group, the trend suggests that the effects of action potentials driven orthodromically without mGluR activation are similar to the detrimental effects of action potentials driven antidromically. It appears that activation of mGluR receptors keeps neurons healthy in the face of potentially damaging levels of synaptic drive.

2.7. Possible Role of mGluRs

How could mGluR activation work to maintain the health of NM neurons? One attractive possibility is in the control of calcium homeostasis. The results of a series of beautiful experiments by Zirpel *et al.* (1995a; Zirpel and Rubel, 1996) coincide quite well with the Y10B results described above. They have shown that afferent activity regulates the intracellular Ca^{2+} concentration of NM neurons and that mGluR activation plays an important role controlling these Ca^{2+} levels. First, they found that intracellular Ca^{2+} levels in NM rise within one hour following deafferentation. The rise in intracellular Ca^{2+} concentration in quiescent cells is somewhat surprising because it is usually assumed that action potentials should increase Ca^{2+} concentrations because they would open voltage-dependent Ca^{2+} channels. Thus, when action potentials are eliminated by deafferentation, one would expect that there would be lowered Ca^{2+} influx and consequently lower intracellular Ca^{2+} levels. Zirpel *et al.* (1995a; Zirpel and Rubel, 1996) found the opposite result. In a slice preparation, the rise in Ca^{2+} levels that occurs after deafferentation was prevented by orthodromic stimulation. Antidromic stimulation, on the other hand, tends to accentuate the rise in Ca^{2+} levels in deafferented NM neurons. If one assumes that high levels of intracellular Ca^{2+} is indicative of an unhealthy cell, then these results parallel those using the assays of ribosomal structure and function. Recall that orthodromic activation maintains healthy ribosomes (maintains low Ca^{2+} concentrations) and antidromic activity, if anything, negatively impacts ribosomal integrity (accentuates the deafferentation-induced rise in Ca^{2+} concentrations).

The pharmacological experiments performed by Zirpel and Rubel (1996) also parallel the results of the experiments assaying ribosomal integrity. When slices were stimulated in a medium containing the metabotropic receptor antagonist MCPG, the stimulation did not prevent the rise in intracellular Ca^{2+} levels. In fact, the rise was greater than that observed in unstimulated NM neurons. As discussed above, a similar lack of effect of orthodromic stimulation on Y10B immunoreactivity was observed when the slice was maintained in MCPG. If anything, neurons stimulated in the presence of MCPG showed poorer ribosomal integrity than unstimulated neurons. One difference between the calcium imaging studies and the immunoreactivity studies is that the potential detrimental effects of stimulation in the presence of the metabotropic receptor antagonist were much more robust in the calcium imaging studies. It should be noted, however, that the dose of MCPG used in the immunoreactivity studies was only one quarter of the dose

used for the calcium imaging studies, but even at this lower dose there was a trend towards detrimental effects.

The modulation of intracellular Ca^{2+} concentration by mGluR activation is particularly intriguing because intracellular Ca^{2+} levels have been proposed to play a role in various types of neuronal cell death (Franklin and Johnson, 1992). One rational hypothesis is that the rise in intracellular Ca^{2+} levels may somehow lead to the disruption of ribosomal integrity, and hence, function. A change in ribosomal function appears to be an early step leading to degeneration and atrophy of NM neurons.

The use of mGluR receptors for maintaining the health of NM neurons provides an enchanting example of exquisite biological design. NM neurons are highly active, even in the absence of acoustic environmental stimuli. For instance, *in vivo* recordings have shown that rates of spontaneous activity in NM are in the neighborhood of 100 Hz (Warchol and Dallos, 1990). Such high rates of activity would be expected to place a huge metabolic demand on the neuron and it would not be surprising if prolonged exposure to such a high rate of activity would produce lethal intracellular Ca^{2+} levels. The high rate of spontaneous activity is completely driven by synaptic input and is not the result of properties intrinsic to the NM neuron, as can be seen by the fact that there is no spontaneous activity when the synaptic drive is eliminated by cochlea removal (Born *et al.*, 1991). Thus, the high rate of spontaneous activity is produced by glutamate being released from the active auditory nerve fibers. Here again, high levels of glutamate are typically thought to be neurotoxic in other systems (Choi, 1992). The NM neuron, however, appears to have evolved an elegantly efficient mechanism for dealing with its potentially toxic local environment. The same molecule that has the potential to produce toxicity (glutamate) through activation of one type of receptor (the ionotropic receptor) also activates a second type of receptor (the metabotropic receptor) that results in the maintenance of safe intracellular Ca^{2+} levels.

3. SUMMARY AND CONCLUSIONS

The auditory nerve afferents have two influences on the postsynaptic neurons in the chick cochlear nucleus, nucleus magnocellularis (NM). First, they provide the excitatory drive to NM neurons that leads to the generation of action potentials, and second, auditory nerve activity is needed for maintaining the metabolic integrity of the postsynaptic neuron. It appears that the neurotransmitter glutamate is responsible for both of these functions, but each function is accomplished through the activation of different classes of excitatory amino acid receptors. Activation of the non-NMDA ionotropic receptors is necessary for the fast synaptic transmission required for action potentials, but is not necessary for the afferent regulation of metabolic integrity of these neurons (as judged by changes in antigenicity for a ribosomal epitope). In contrast, activation of metabotropic glutamate receptors has no apparent influence on the moment-to-moment electrical activity of NM neurons, but is required for the preservation of metabolic integrity.

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7

THE DEVELOPMENTAL INFLUENCE OF INHIBITORY SYNAPTIC TRANSMISSION

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1. INTRODUCTION

Environmental manipulations that affect brain development form the primary evidence for "activity-dependent" mechanisms. Manipulations of this sort probably alter the normal amount or the pattern of synaptic transmission and action potentials, and this altered activity state somehow influences the growth and differentiation of synaptic connections. The first studies to draw a strong causal relationship between environmental stimulation and the development of connections were performed in the cat visual system (Wiesel and Hubel, 1963, 1965; Hubel and Wiesel, 1965). In control animals, extracellular recordings from cortex show that most neurons fire action potentials in response to stimulation of either eye. However, when visual stimulation to one eye is decreased during development, there is a dramatic loss in the ability of that eye to activate cortical neurons. While these changes are striking, the cellular mechanisms whereby differences in neural activity are translated into structural or functional changes are far from clear. To date, most studies have focused on the cellular mechanisms that might operate at developing excitatory synapses, such as the nerve-muscle junction. In contrast, little is known about the maturation of inhibitory synaptic contacts, and whether they too can exhibit "activity-dependent" mechanisms. This chapter summarizes experimental findings from a developing inhibitory projection in the auditory system, and provides evidence that inhibitory transmission plays an active role during ontogeny.

There are a number of experiments suggesting that electrical activity does influence the developing auditory system. Two general strategies have been used. First, one can decrease the amount of sound stimulation, thus decreasing the amount of driven activity, over a period of animals' development. Second, one can rear animals in an artificial acoustic environment, thus imposing a specific pattern of driven activity on auditory neurons. An example of the first approach involves raising chicks with bilateral ear plugs and subsequently testing their ability to discriminate between distinct sound frequencies

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using a behavioral assay (Kerr *et al.*, 1979). The results show that animals are poorer at discriminating between sound frequencies even though the cochlea appears to function normally, suggesting that central auditory processing is modified. An example of the second approach involves rearing mice in an acoustic environment of repetitive clicks. These click-reared mice are subsequently found to have single-neuron frequency tuning curves that are significantly broader than those of control animals (Fig. 1A). Despite the difference in tuning curves, absolute thresholds for sound are equal in the experimental and control groups of mice (Sanes and Constantine-Paton, 1985). Interestingly, a similar finding has been reported in neonatally deafened cats if one ear is stimulated with repetitive current pulses delivered through an implanted electrode, and compared to unstimulated deafened cats (Snyder *et al.*, 1990). To evaluate neural function in the deaf animals, one can measure the amount of midbrain tissue that is activated by an electrical stimulus to one position in the cochlea. These "spatial" tuning curves are significantly broader in pulse-reared deafened animals compared to those obtained from deafened, unstimulated animals (Fig. 1B).

One simple hypothesis to account for both types of altered tuning curves postulates that stimulated auditory neurons retain a slightly greater assortment of synaptic inputs than do auditory neurons developing with normal patterns of activity. That is, synapse elimination is thought to occur when some afferents are silent while others are active. Click- or pulse-rearing effectively prevents this from occurring. These results, and many others of the same variety (Silverman and Clopton, 1977; Moore and Irvine, 1981; Gottlieb, 1980a, 1980b, 1982, 1983; Knudsen *et al.*, 1984; King *et al.*, 1988; Withington-Wray *et al.*, 1990; Poon *et al.*, 1990; Poon and Chen, 1992; Mogdans and Knudsen, 1992; Wilmington *et al.*, 1994; Schnupp *et al.*, 1995), suggest that neural activity plays an important role in the maturation of central auditory function, and motivates our search for the underlying cellular mechanisms.

2. KEY QUESTIONS

How does neural activity alter the developing nervous system? The first point to be made is that the possibilities are many. Neurotransmission has been shown to influence a wide range of developmental events, including neuron survival, process outgrowth, and the expression of ion channels and receptors. Most studies have focused on the effects of excitatory synaptic transmission, and the great majority of these have been performed at the nerve-muscle junction or at retinal ganglion cell synapses in the midbrain (frogs) and thalamus (mammals). These studies generally assume that alterations in the number, or the strength, of excitatory connections can explain how environmental stimulation influences brain development.

While there is excellent evidence that excitatory synapses can be modified by experience, there are many other types of synapses in the brain that might also be changing. Inhibitory synapses probably make up the second largest group of connections in the CNS. These terminals typically release glycine or γ-aminobutyric acid (GABA) and hyperpolarize postsynaptic neurons by gating open either chloride or potassium channels. Changes in neuron excitability have traditionally been explained by enhancement or depression of excitatory synapses, but such changes could just as easily be due to equal and opposite changes in synaptic inhibition. For example, monocular deprivation almost certainly leads to a decrease in the number or strength of excitatory connections in the visual cortex, but what is the formal proof that inhibition is unaffected?

At first, we wanted to know whether inhibitory synapses were just along for a developmental ride, or whether inhibitory transmission is necessary for normal maturation to occur. Since the loss of excitatory synapses often causes postsynaptic neurons to atrophy or die, particularly during development, we began by asking: Does inhibitory transmission exert a trophic influence on the maturation of postsynaptic structure? We next turned out attention to the strength and maintenance of synaptic connections: Does the strength of inhibitory synapses depended on transmission at these contacts? Do inhibitory synapses influence the development of excitatory synaptic function? If inhibitory transmission contributes to the development of central auditory function, what are the underlying cellular mechanisms at these synapses?

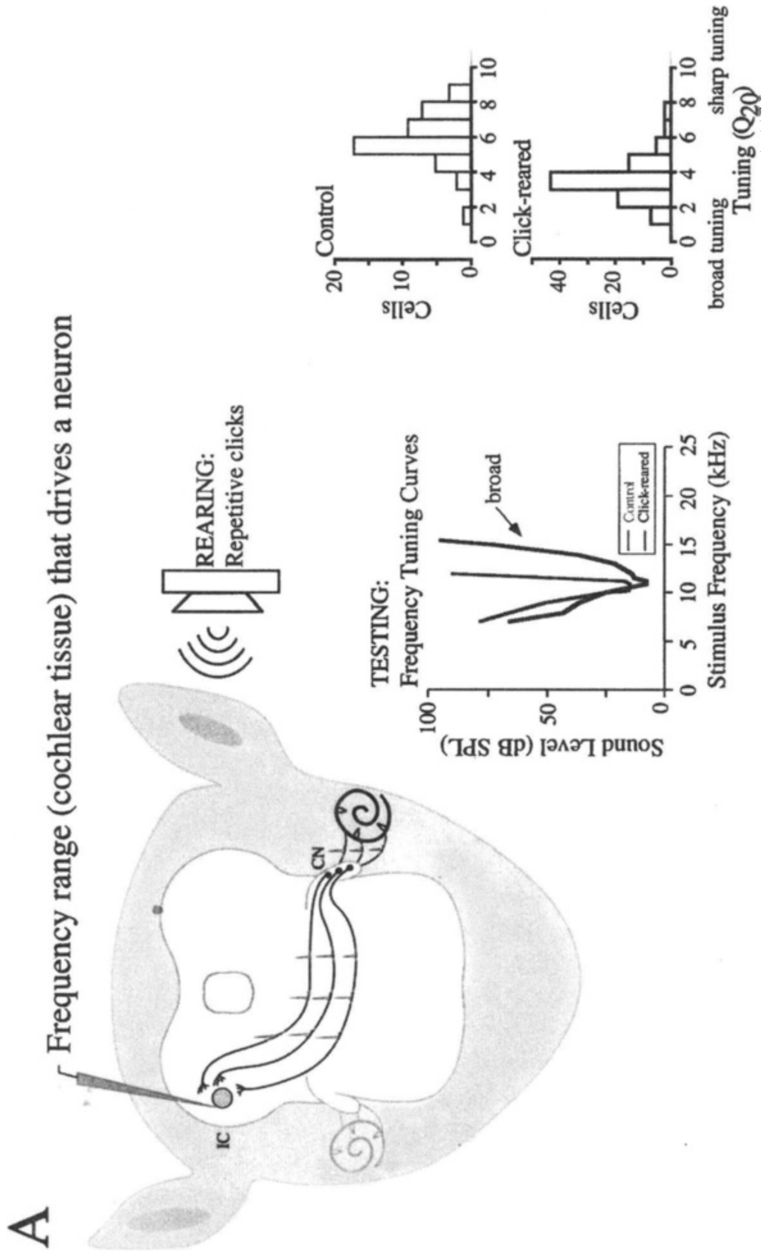
3. A MODEL SYSTEM FOR INHIBITORY DEVELOPMENT

One reason that motor neuron and retinal ganglion cell afferents have been so well studied is that they form pure excitatory projections. Motor neuron synapses release acetylcholine to depolarize muscle cells, and retinal ganglion cell synapses release glutamate to depolarize midbrain or thalamic neurons. Manipulations of the spinal motor nerve, or the optic nerve, lead to changes in excitatory synaptic transmission at the first postsynaptic target (e.g., muscle and midbrain, respectively). To selectively test the role of inhibitory transmission during development, it seemed important to find a similar kind of projection. Yet, most inhibitory synapses in the mammalian brain come from interneurons (Eccles, 1969), making it extremely difficult to stimulate them selectively and subject them to experimental manipulation.

As it turns out, the central auditory system is rife with inhibitory projections, and we have made use of one such pathway in the gerbil (*Meriones unguiculatus*) to study the development and plasticity of inhibitory connections. The gerbil auditory system develops rapidly, responding to airborne sound by about postnatal day 12, and reaching functional maturity between postnatal days 16 to 30, depending on the response property being considered (Finck *et al.*, 1972; Ryan and Woolf, 1988; Harris and Dallos, 1984; Woolf and Ryan, 1984, 1985).

3.1. LSO and Sound Localization

In the auditory brain stem there is a particularly favorable group of neurons, called the lateral superior olivary nucleus (LSO), that receive excitatory projections driven by the ipsilateral ear and inhibitory projections driven by the contralateral ear (Boudreau and Tsuchitani, 1970). As shown in Fig. 2, this conformation permits LSO neurons to respond selectively to differences in sound level at the two ears, usually referred to as interaural level differences (ILD). When the sound is on the ipsilateral side of the head (Fig. 2, top left), then the excitatory pathway is maximally activated by sound. Since the head acts as a baffle to decrease sound level at the contralateral ear (when the sound frequency wavelength is smaller than the width of the head), the inhibitory pathway is minimally activated by the sound source. In this case, the postsynaptic neuron adds up much excitation and little inhibition, producing a large discharge rate. When the sound is on the contralateral side of the head (Fig. 2, top right), then the inhibitory pathway is maximally activated, the excitatory pathway is minimally activated, and the postsynaptic neuron produces a small rate of discharge. Most mammals make use of ILD cues to determine the location of a sound source along the horizon, and behavioral experiments


A
Frequency range (cochlear tissue) that drives a neuron
IC
CN
REARING:
Repetitive clicks
TESTING:
Frequency Tuning Curves
broad
Control
Click-reared
Sound Level (dB SPL)
Stimulus Frequency (kHz)
Control
Click-reared
Cells
broad tuning
sharp tuning
Tuning (Q_{20})

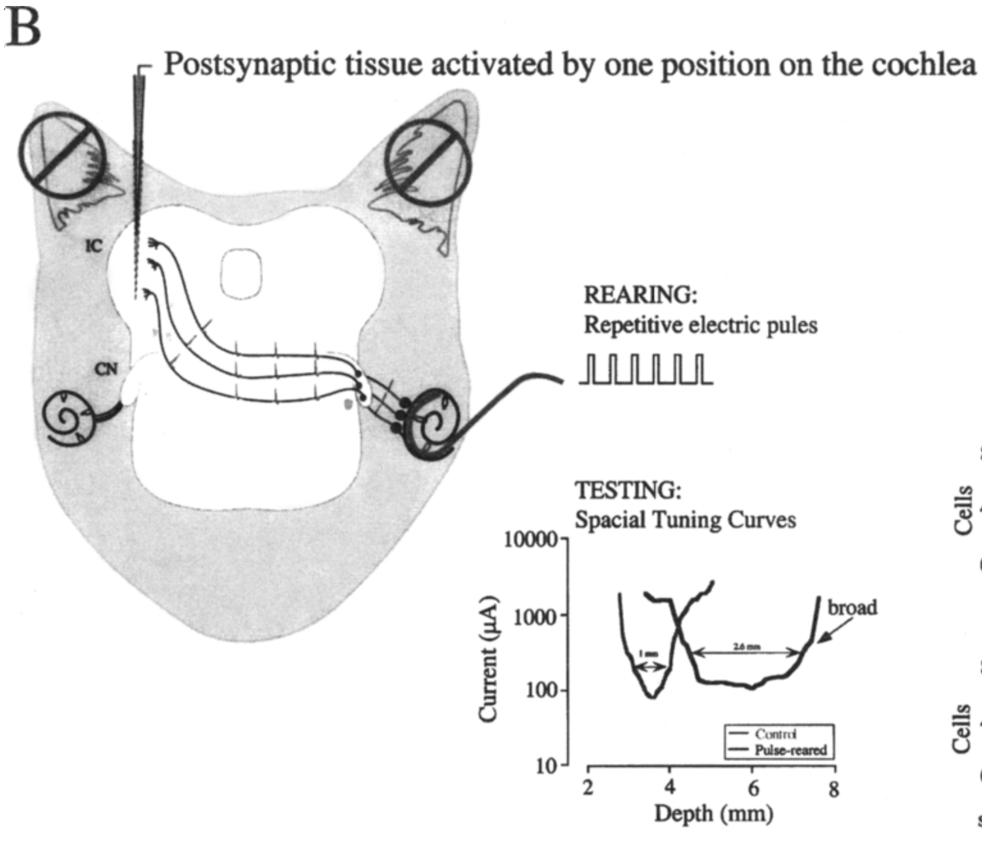


Figure 1. Synchronizing activity in the developing auditory system produces broad tuning. (A) In one experiment, mice were rearing in an acoustic environment of repetitive clicks. The schematic (left) illustrates that these brief broadband stimuli synchronized the activity of eighth nerve fibers. When frequency tuning curves were obtained from single neurons in the inferior colliculus, they were found to be broader (center). As shown by the summary histogram (right), the average width of tuning curves from control values (Q_{20}) was much sharper for control neurons with a characteristic frequency of 12 kHz compared to those recorded from click-reared animals. Adopted from Sanes and Constantine-Paton (1985). (B) In a second experiment, neonatally deafened kittens were implanted with cochlear prostheses as juveniles, and reared with repetitive electrical pulses. The schematic (left) illustrates that these brief stimuli synchronized the activity of eighth nerve fibers. When spatial tuning curves were obtained by recording through the entire depth of the IC, they were much broader (center). As shown by the summary histogram (right), the average width of tuning curves from control values (Tuning) was much sharper for control colliculi compared to those from pulse-reared animals. Adopted from Snyder *et al.* (1990).

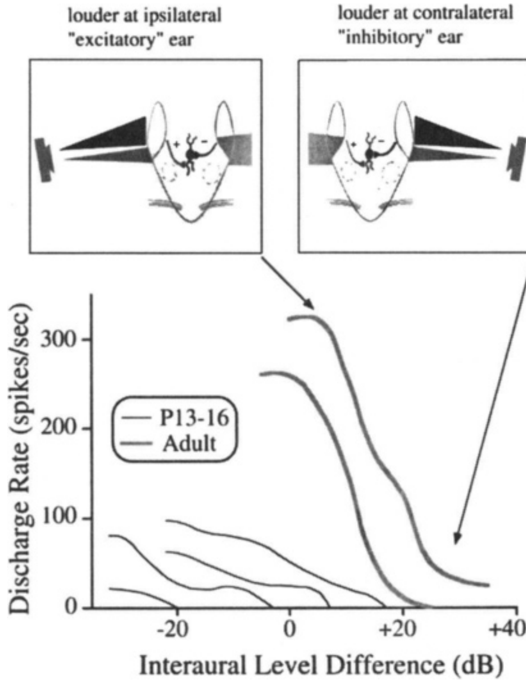


Figure 2. Development of level difference coding. (Top) The schematics illustrate that single neurons that are excited by the left ear and inhibited by the right ear are able to encode interaural sound level differences. (Bottom) Interaural level difference functions recorded from single neurons in the gerbil lateral superior olivary nucleus. The functions from young animals are shallower and are somewhat irregular in shape. Negative values denote greater intensity to the ipsilateral ear. Adult curves have a large dynamic range. Adopted from Sanes and Rubel (1988).

show that gerbils can localize sound cues through level differences, with a best resolution of about 14 degrees (Heffner and Heffner, 1988).

A plot of ILD versus discharge rate for single LSO neurons is shown in Fig. 2 (bottom). In the adult gerbil, LSO neurons change their discharge rate by several hundred spikes/sec as ILD varies by about 30 dB. In juvenile animals, the situation is quite different. Single LSO neurons have a relatively poor resolution (i.e., Δdischarge/ΔILD), primarily because the peak discharge rate is so low (Sanes and Rubel, 1988). In addition, some neurons appear to have very irregular responses as ILD is altered. Similar observations have been made in the kitten inferior colliculus (Moore and Irvine, 1981). Developmental changes in the inhibitory pathway may contribute to the maturation of this circuit. For example, ILD functions shift about +10 dB during development (Sanes and Rubel, 1988), suggesting that the relative strength of contralateral inhibition may be changing during postnatal development. In fact, we have also observed a transformation of the transmitter system that mediates contralateral inhibition. Glycine receptor expression is at first uniform within the LSO, but assumes an inhomogeneous distribution pattern during the first three postnatal weeks (Sanes and Wooten, 1987). Thus, our survey of system level properties in LSO led us to believe synaptic inhibition was somewhat dynamic during development.

3.2. Development of Synaptic Function in LSO

To examine the development of synaptic inhibition more directly, intracellular recordings were obtained from LSO neurons in a brain slice preparation. As shown in Fig. 3A, a major advantage of the LSO is the presence of an independent excitatory projection from the ipsilateral cochlear nucleus (CN), and a glycinergic inhibitory projection from the medial nucleus of the trapezoid body (MNTB) which is normally activated by

the contralateral ear (Rasmussen, 1946; Morest, 1968; Browner and Webster, 1975; Warr, 1982; Moore and Caspary, 1983; Glendenning *et al.*, 1985; Spangler *et al.*, 1985; Cant and Casseday, 1986; Zook and DiCaprio, 1988). Both the ipsilateral and the contralateral afferent projections form topographic maps of frequency in the LSO, with low frequencies represented ventrolaterally and high frequencies represented dorsomedially (Sanes *et al.*, 1989).

When brief electric stimuli are delivered to an electrode placed on the ipsilateral afferent pathway (Fig. 3A, excitatory), we commonly observe evoked excitatory postsynaptic potentials (EPSPs) in LSO neurons. In contrast, when electric stimuli are delivered to the MNTB (Fig. 3A, inhibitory), we usually observe evoked inhibitory postsynaptic potentials (IPSPs). As shown in Fig. 3B, the duration of EPSPs and IPSPs decreases dramatically during the course of development. For example, the maximum IPSP duration declines from about 62 ms at P1–7 to about 8 ms at P17–23 (Sanes, 1990,

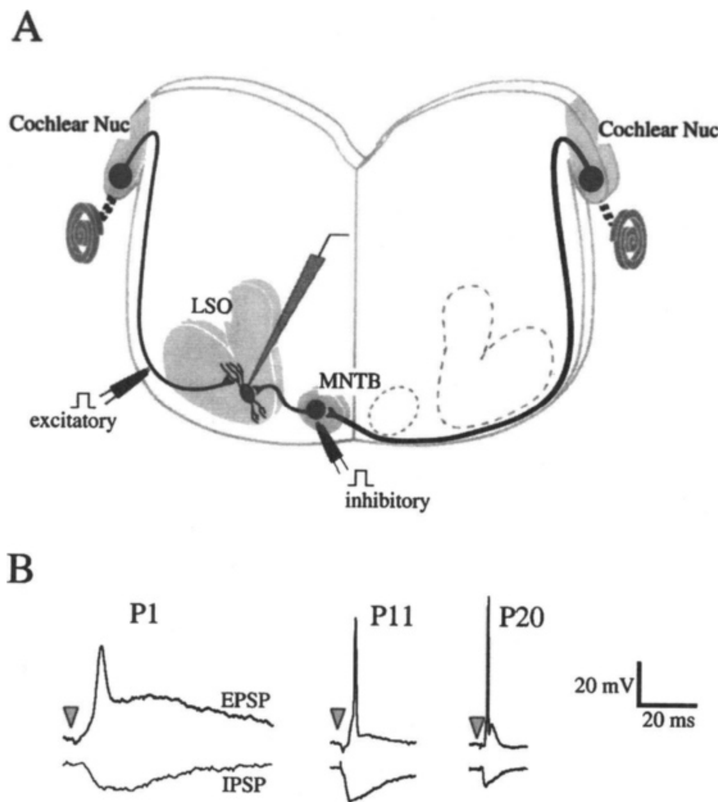


Figure 3. Schematic of LSO brain slice preparation to study development of synaptic inhibition. (A) The schematic shows the key afferent pathways to the lateral superior olivary nucleus (LSO). On the ipsilateral side, the cochlear nucleus provides a direct excitatory projection to the LSO. On the contralateral side, the cochlear nucleus projects across the midline to the medial nucleus of the trapezoid body (MNTB). The MNTB cells provide a direct glycinergic projection to the LSO. Stimulating and recording electrodes are indicated. (B) Representative excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs, respectively) recorded intracellularly from single LSO neurons. At birth, the synaptic potentials evoked by a single stimulus pulse to either the ipsilateral (EPSP) or contralateral (IPSP) pathway are extremely long-lasting. The duration of synaptic potentials decreases by ·10 times during the first 3 weeks of development.

1993). The prolonged IPSPs could be due to any number of immature properties including an inefficient glycine uptake system (Zukin *et al.*, 1975), the expression of neonatal glycine receptors (Becker *et al.*, 1988; Takahashi *et al.*, 1992), immature passive membrane properties (Sanes, 1993), or the physical location of inhibitory synaptic terminals (Cant, 1984).

An estimate of the number of MNTB afferents that innervate a single LSO neuron was estimated by obtaining a large family of MNTB-evoked IPSPs, and calculating the area between each IPSP and the resting baseline. The basic assumptions are that each axon will evoke a PSP when stimulated, and that the PSPs will summate in discrete steps as the additional fibers are recruited by the electric stimulus (Redfern, 1970). Therefore, discrete increments in PSP size serve as a measure for the number of axons making functional contact with a single postsynaptic cell. Using this analysis, we found an apparent change in the number of MNTB afferents per LSO neuron during the first 3 postnatal weeks, declining from 16 at P1–7 to about 9 at P17–23 (Sanes, 1993). A complementary observation is that single MNTB terminal arborizations in the LSO become physically restricted during development (Sanes and Siverls, 1991). At P12–13, MNTB terminals contained an average of 244 boutons, and these boutons spread 121 μm across the LSO frequency axis. Each of these values declined significantly by P18–25 (Number of boutons = 158; Bouton spread = 93 μm). Together, these findings suggest that inhibitory function is changing during the first few postnatal weeks, and there is a physical alteration in the pattern of innervation. These finding led us to ask whether inhibitory transmission might be necessary for the normal maturation of structure and function in the LSO.

4. TROPHIC INFLUENCE OF SYNAPTIC INHIBITION

Decreasing excitatory transmission typically leads to degenerative changes in postsynaptic neurons, particularly during development (Larsell, 1931; Levi-Montalcini, 1949; Globus and Scheibel, 1966; Peusner and Morest, 1977; Webster and Webster, 1979; Parks, 1979). These effects appear to be at least partially attributable to the transmitter-evoked regulation of postsynaptic protein metabolism (Steward and Rubel, 1985; Born and Rubel, 1988; Hyson and Rubel, 1989). Dendritic arborizations are also adversely affected by excitatory denervation (Feng and Rogowski, 1980; Parks, 1981; Trune, 1982), and these changes can occur within 30 mins of denervation (Deitch and Rubel, 1984). To investigate the influence of inhibitory synaptic transmission on the development or maintenance of neural elements, we performed a series of experimental manipulations that were intended to decrease inhibitory transmission in the LSO during the period when dendrites and axonal arbors are reaching a mature phenotype.

4.1. Effect of Inhibitory Transmission on LSO Dendrites

The dendritic fields of LSO neurons go through a period of refinement during the first three postnatal weeks, and two critical changes occur (Sanes *et al.*, 1992a). First, the number of branch points decrease significantly from P12 to P21, particularly in the high frequency region. Second, the dendritic arbors of high frequency neurons decrease their arbor span along the frequency axis by about 40% during the third postnatal week. Interestingly, this dendritic refinement is well-correlated with a refinement of frequency tuning

(Sanes *et al.*, 1992a). Therefore, both LSO dendritic arborizations and MNTB afferent arborizations undergo a concomitant modification of morphology.

We examined the trophic influence of inhibitory synaptic transmission *in vivo* by using two manipulations to attenuate glycinergic synaptic activity in the LSO during development (Fig. 4A). A principal reason for choosing the MNTB projection to the LSO as a model for inhibitory development is its unique geometry, which allows for selective inactivation of this pathway. Therefore, we first surgically extirpated the contralateral cochlea at P7 to functionally denervate the afferents from the MNTB to the LSO (Sanes *et al.*, 1992b; Aponte *et al.*, 1996). Second, strychnine-containing continuous release pellets were implanted at P3 to decrease the level of glycinergic transmission, albeit throughout the nervous system (Sanes and Chokshi, 1992). Both manipulations began before the onset of sound-evoked activity, and the effect on LSO dendrites was assessed morphometrically by comparison with age-matched controls. The effects of each

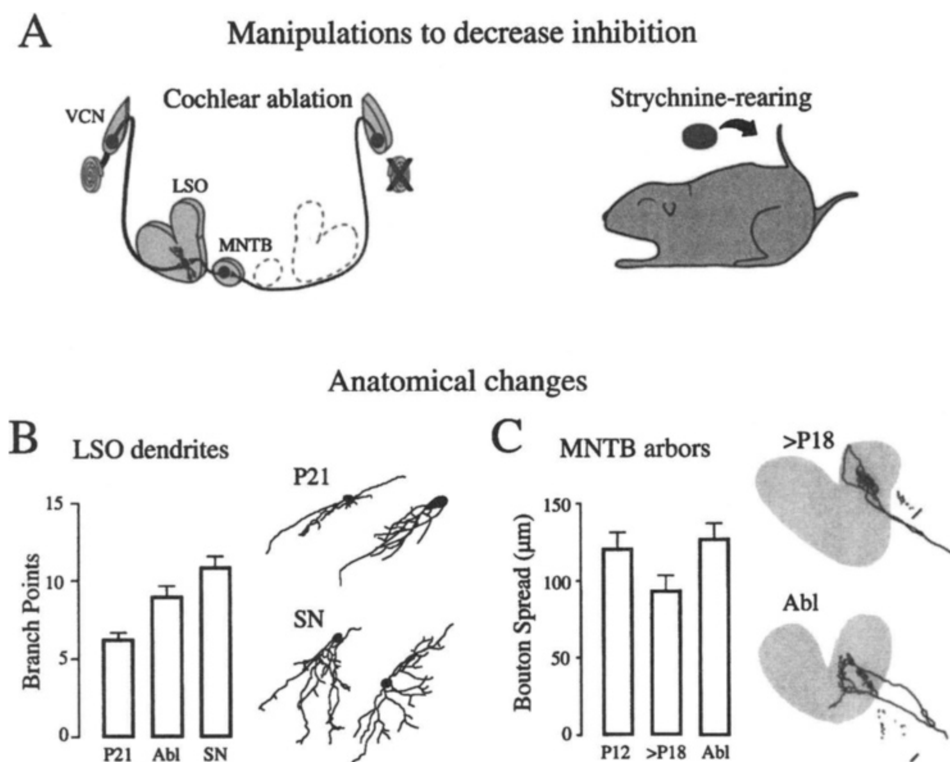


Figure 4. Manipulations to decrease inhibitory transmission influence the development of neuron form. (A) Two manipulations were used *in vivo* to attenuate inhibitory transmission in the LSO. First, unilateral cochlea removal (left) at postnatal day 7 functionally denervated MNTB neurons projecting to the contralateral LSO. Second, implantation of a time release strychnine (SN) containing pellets (right) at postnatal day 3 led to a general attenuation of glycinergic transmission. (B) When Golgi stained LSO neurons were analyzed at P21, and it was found that either manipulation led to a large increase in dendritic branch points. Representative neurons are shown from a control P21 animal and a SN-reared animal. Adopted from Sanes *et al.*, (1992a) and Sanes and Chokshi (1992) (C) When single MNTB terminal arborizations in the LSO were analyzed at P12–13 and P18–25, it was found that they became more refined across the LSO tonotopic axis. Following contralateral cochlea ablation, this refinement failed to occur. Representative MNTB arbors and the bouton distributions are shown for a control fiber (>P18) and a denervated fiber (Abl). Adopted from Sanes and Takacs (1993).

manipulation are nearly identical. LSO dendrites have more branches and are more spread out along the tonotopic axis of LSO compared to control animals (Fig. 4B). Interestingly, this effect seems to be most prominent in the medial (high frequency) region of the LSO, the same area that receives the major fraction of MNTB axons and contains the highest density of glycine receptors (Sanes *et al.*, 1987; Sanes and Siverls, 1991).

Both manipulations unintentionally produce systemic effects that hamper interpretation. For example, cochlear ablation induces sprouting in the superior olivary complex (Kitzes *et al.*, 1995; Russell and Moore, 1995), and strychnine treatment undoubtedly blocks glycinergic synapses throughout the central auditory system. To provide a more direct test of the idea that glycinergic synapses influence dendrite growth, the MNTB and LSO were placed in organotypic culture, and exposed to normal culture media or SN-containing media (Fig. 5). As predicted by our *in vivo* observations, SN-treatment results in LSO dendrites that have a significantly greater number of branches compared to control neurons (Sanes and Hafidi, 1996). While tissue culture experiments travel with their own set of caveats, the *in vivo* and *in vitro* findings, together, provide argue strongly that spontaneous or evoked inhibitory transmission does influence the normal maturation of dendrite structure in the LSO.

4.2. Effect of Inhibitory Transmission on MNTB Terminals

As mentioned above, our descriptive studies suggest that some inhibitory synapses are eliminated during the course of development. Since the maintenance and elimination

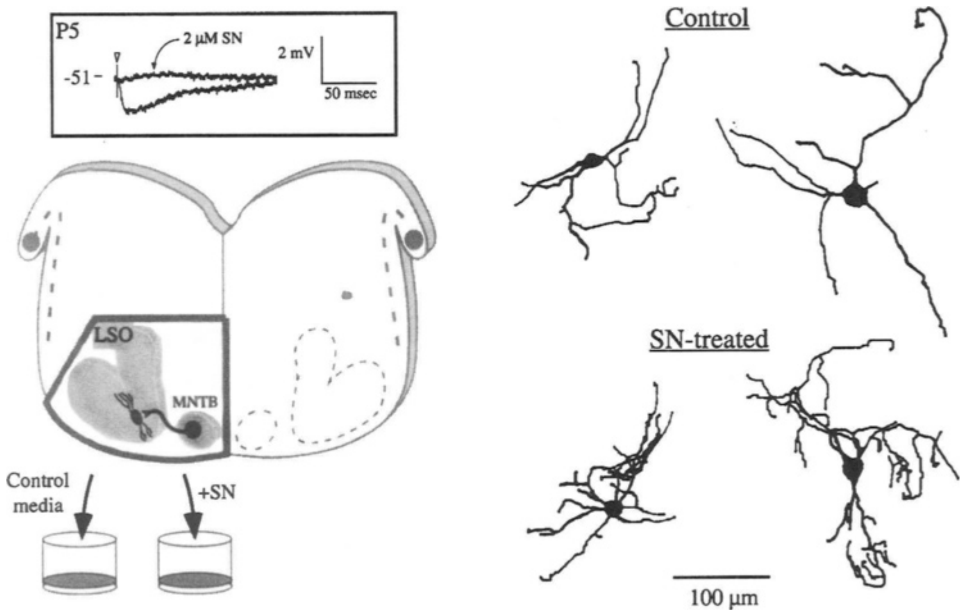


Figure 5. Organotypic cultures containing the LSO and MNTB from P6–8 day animals were grown *in vitro* for about one week, either in normal media or SN-containing media (left). When neurons were filled with biocytin and analyzed (right), it was found that the SN-treated cells contained about 3 times as many branches compared to controls. The inset shows that at the time of culturing, LSO neurons display MNTB-evoked IPSPs, and these are attenuated with 2 µM SN. Adopted from Sanes and Hafidi (1996).

of excitatory terminals has been linked experimentally to the activity at these synapses (Udin, 1983; Reh and Constantine-Paton, 1985; Sretavan *et al.*, 1988; Stryker and Harris, 1986), we asked whether the specificity of MNTB terminals is also dependent on a functional connection to the cochlea. As before, the contralateral cochlea was ablated at P7 (Fig. 4A) and single MNTB neurons were subsequently filled (at P18–25) with horseradish peroxidase in a brain slice preparation such that terminal arbors in LSO could be drawen under a microscope and measured (Sanes and Takacs, 1993).

As shown in Fig. 4C, functional denervation of MNTB neurons results in a failure to attain the normal level of anatomical specificity. Terminal boutons spread the same distance across the tonotopic axis as is found in control animals at P12–13. Even though the manipulation functionally denervates MNTB cell bodies, the arbors do not display signs of atrophy. Both the total axonal length within the LSO boarders, and the number of boutons per MNTB fiber were similar in control and ablated animals. Thus, inhibitory MNTB arbors do not go through their normal period of anatomical refinement when denervated, suggesting that inhibitory activity also plays a role in the maintenance or stabilization of inhibitory synaptic contacts.

4.3. Effect of Inhibitory Transmission on LSO Function

A chronic reduction in neural transmission during development is often associated with a decrease in synaptic efficacy. As discussed above, this has been clearly illustrated in the visual cortex: When retinal activity is decreased during development, it is found that the majority of visual cortex neurons no longer respond to stimulation of the deprived eye (Wiesel and Hubel, 1963). Subsequent experiments suggest that synaptic strength is regulated by competition between active and inactive terminals (Hubel and Wiesel, 1965; Constantine-Paton *et al.*, 1990; Shatz, 1990), and by non-competitive interactions (Watkins *et al.*, 1978; Dubin *et al.*, 1986; for review, see Sherman and Spear, 1982). Although these studies were conducted with extracellular electrodes, and did not explicitly examine the relative effect on individual excitatory or inhibitory connections, intracellular recordings from motor neurons suggest that maintenance of synaptic strength is a use-dependent phenomenon (Eccles *et al.*, 1959). Thus, the present study was designed to evaluate whether our manipulations to attenuate inhibitory transmission had an effect on the strength of synaptic transmission in the developing LSO.

Following a brief period of reduced inhibitory transmission (Fig. 4A), the strength of excitatory and inhibitory synapses was examined in a brain slice preparation (Kotak and Sanes, 1996). Whole-cell recordings were made from LSO neurons, and the stimulus-evoked IPSPs or EPSPs (as shown in Fig. 3A) were analyzed. The percentage of LSO neurons exhibiting MNTB-evoked IPSPs is reduced significantly in both ablated and SN-treated animals (only 1 of 11 SN-treated neurons had an MNTB-evoked IPSP). In those neurons that do have MNTB-evoked IPSPs, the amplitude is significantly reduced, and this decrease is accompanied by a 8 mV depolarization in the IPSP reversal potential (Fig. 6A). This result, then, resembles previous findings on disuse, although the mechanisms require further consideration (see below).

A surprising finding was made when we examined the unmanipulated ipsilateral pathway. Ipsilaterally-evoked EPSPs are of unusually long duration in experimental animals (Fig. 6B). Furthermore, these long duration EPSPs are significantly shortened by exposing the neuron to AP-5, an NMDA receptor antagonist. Thus, inhibitory transmission regulates the functional expression of at least one postsynaptic protein (the NMDA receptor), thereby controlling the strength of excitatory transmission. Inhibitory

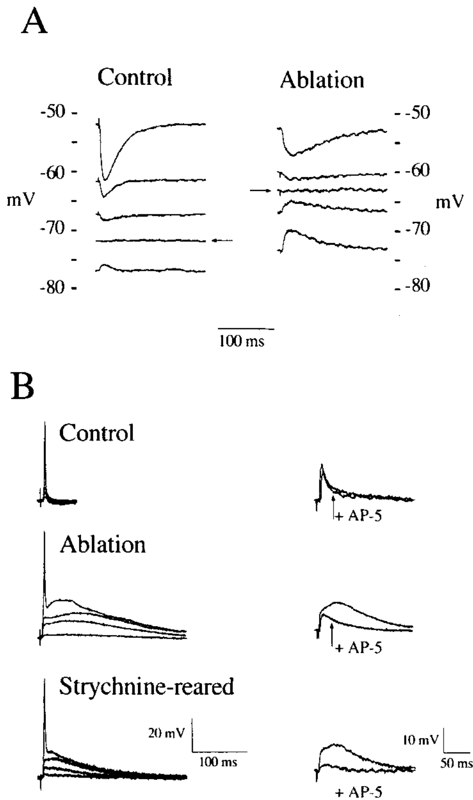


Figure 6. Manipulations to decrease inhibitory transmission influence synaptic strength. (A) Following contralateral cochlear ablation, intracellular recordings were obtained from LSO neurons. The average size of MNTB-evoked IPSPs was smaller in ablated animals, and the reversal potential of these IPSPs was more depolarized by about 8 mV (arrows). Only 1 of 10 neurons in SN-reared animals displayed MNTB-evoked IPSPs. (B) When the unmanipulated ipsilateral pathway is stimulated in control animals, brief EPSPs and an action potential result. In contralaterally ablated and SN-reared animals, the ipsilateral pathway produced long-lasting EPSPs. The long-lasting EPSPs were largely eliminated by AP5, indicating that they resulted from NMDA receptor expression. Adopted from Kotak and Sanes (1996).

transmission may effect the maturation of calcium channels as well. Neurons from ablated or SN-treated animals display broad rebound depolarizations following membrane hyperpolarization, and these are abolished in the presence of Ni^{2+}.

Since both cochlear ablation and strychnine-rearing were initiated prior to the onset of sound-evoked activity (SN-rearing began at P3, Ablations performed at P7), the results suggest that *spontaneous* inhibitory transmission influences the development of postsynaptic properties. As summarized in Fig. 7, inhibitory disuse leads to homosynaptic depression of the inhibitory terminals and heterosynaptic enhancement of the ipsilateral excitatory terminals.

5. POSSIBLE MECHANISMS OF INHIBITORY PLASTICITY

The cellular mechanism(s) by which inhibitory synapses regulate postsynaptic shape remain unknown, and the following experimental observations only suggest some possibilities. One of the first mysteries to arise is how a small amount of spontaneous activity can have such a great effect on neuronal maturation. Single unit recordings from the embryonic rat retina demonstrate that neurons have an average discharge rate of 0.9 spikes/sec, but may fire short bursts of up to 100 spikes/sec (Galli and Maffei, 1988; Meister *et al.*, 1991). Similarly, regular bursts of spontaneous discharge occur in the embryonic chick cochlear nucleus (Lippe, 1994). Our own recordings from single auditory midbrain neurons in anesthetized P9–13 gerbils indicate that action potentials are, indeed, the rare event (Kotak and Sanes, 1995). The average discharge rate of all isolated

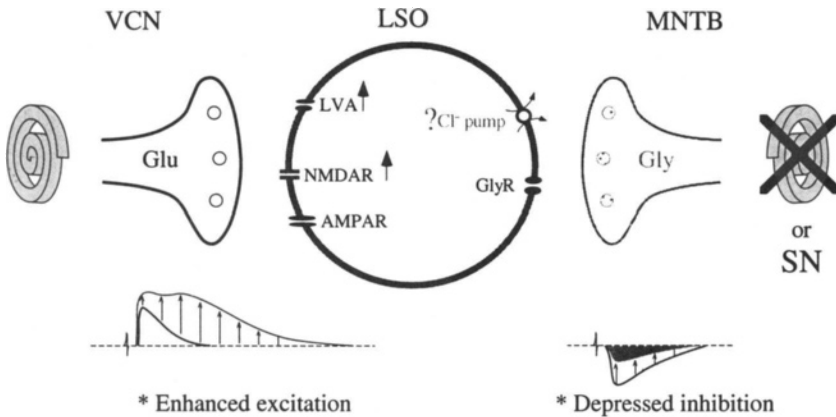


Figure 7. Summary of postsynaptic changes resulting from decreased inhibitory transmission. An LSO neuron is shown to be innervated by a glutamate-containing terminal from the VCN, and a glycine-containing terminal from the MNTB. The manipulations result in enhanced excitatory potentials (left), largely due to addition of functional NMDA receptors (NMDA-R), although new low voltage activated Ca^{2+} channels (LVA) may also contribute. These manipulations also results in decreased inhibitory potentials (right), largely from a change in the chloride equilibrium potential, and possibly due to a decrease in the activity or expression of a chloride transporter (Cl^-).

neurons was 0.4 spikes/sec, and the mean peak discharge rate (i.e., based on the minimum interspike interval) was 37 spikes/sec. It is possible that discharge rates are much higher in the unanesthetized animal, or perhaps many subthreshold events go unreported. However, it seems surprising that so little activity has such a large impact in the developing system.

5.1. Gain Control of Spontaneous and Evoked Activity

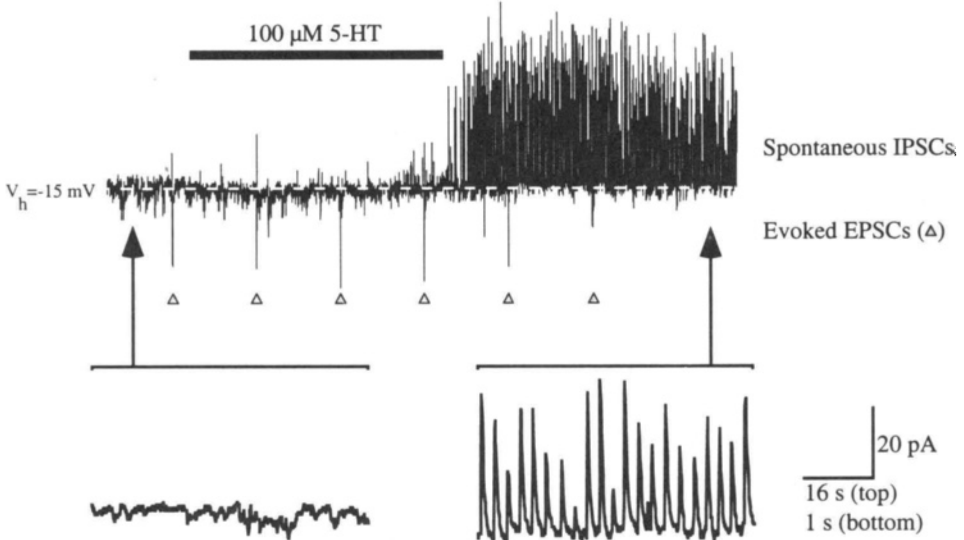
Since alterations of synaptic activity in the gerbil LSO during postnatal development produce profound changes in synaptic efficacy and morphology, we examined serotonergic modulation of synaptic transmission in this nucleus (Fitzgerald and Sanes, 1999). Serotonin-containing cell bodies of the raphe are found just medial to the MNTB, and immunohistochemical staining shows that MNTB and LSO contain 5-HT-positive puncta during the early postnatal period. As shown in Fig. 8A, voltage clamp recordings from LSO neurons in the brain slice preparation demonstrated that exposure to 5-HT increased the frequency of spontaneous inhibitory postsynaptic currents (IPSCs), and substantially depressed evoked excitatory postsynaptic currents (EPSCs).

These results suggest that 5-HT may be an important modulator of synaptic transmission during development. Figure 8B provides a speculative model of how raphe neurons might control the gain of excitatory and inhibitory synaptic activity during development. At this point, the key questions are how the activity of raphe neurons, themselves, are regulated during development, and whether raphe projections to LSO and MNTB can significantly alter the stability of synaptic terminals in the developing LSO.

5.2. Inhibition Regulates Intracellular Calcium and pH

Having shown that inhibitory synaptic activity influences the development of neuronal form and function, we wished to identify the cellular mechanism(s) whereby

A



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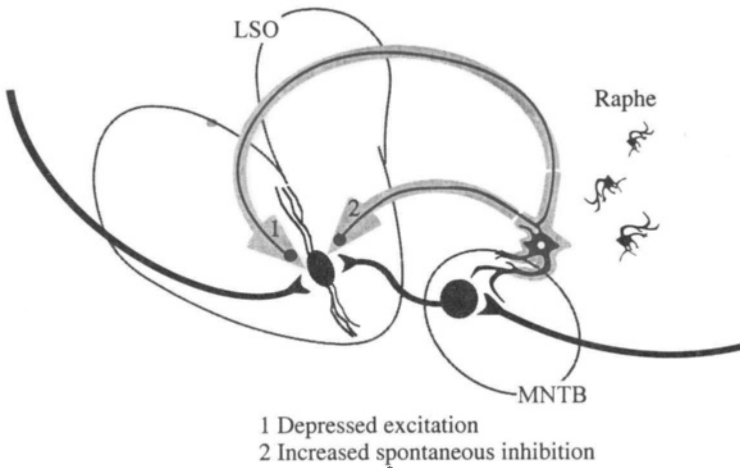

Figure 8. Serotonin (5HT) changes the gain of excitatory and inhibitory synapses in the developing LSO. (A) Superfusion of 5HT while recording from an LSO neuron in voltage clamp leads to a dramatic increase in spontaneous inhibitory synaptic currents (upward deflections), and a decrease in the size of ipsilaterally-evoked excitatory synaptic currents (downward deflections, triangles). A section of the trace before and after 5HT treatment is expanded below. (B) The schematic shows a hypothetical projection from the raphe to the LSO, indicating the presynaptic modulation of terminals could change the gain of synaptic transmission.

inhibitory terminals could control pre- and postsynaptic maturation. There seem to be two leading candidates for a transduction pathway. First, many studies have shown that inhibitory transmitters regulate intracellular free Ca^{2+}, particularly during development (Connor *et al.*, 1987; Obrietan and van den Pol, 1995). MNTB stimulation produces depolarizing IPSPs during the first postnatal week in the rat LSO due to elevated intracellular chloride (Kandler and Friauf, 1995; Kakazu *et al.*, 1999). These depolarizing potentials are likely to produce calcium transients. Synaptic inhibition might also prevent calcium transients that would normally accompany excitatory synaptic activity by introducing a current shunt (Callaway *et al.*, 1995; Lo *et al.*, 1998). An alternative means for inhibition to effect postsynaptic metabolism could involve the regulation of postsynaptic pH. Glycine and $GABA_A$ receptor-coupled channels are permeable to HCO_3^- ions (Bormann *et al.*, 1987), and a transient HCO_3^- conductance may effect both membrane potential and pH (Kaila and Voipio, 1987; Kaila *et al.*, 1993; Staley *et al.*, 1995).

We have examined both of these possibilities in the LSO through the use of fluorescent dyes with a sensitivity to calcium ions or pH (Green *et al.*, 1997). Brain slices at P7–13 were preincubated in the pH-sensitive dye, BCECF-AM, and then exposed to glycine (5 mM, 1 min). As shown in Fig. 9, LSO neurons typically produced an initial alkalinization followed by an longer-lasting acidification. When neurons were preincubated in the Ca-sensitive dye, Fura-2 AM, and then exposed to glycine, they usually displayed a transient calcium influx (Fig. 9B). In separate experiments, LSO membrane potential was recorded during similar glycine exposures (not shown), and most neurons exhibited a brief hyperpolarization followed by a longer-lasting depolarization. A similar set of observations have been made in the auditory midbrain by recording the GABA-evoked change in membrane potential and calcium signal concurrently (Lo *et al.*, 1998). Although preliminary, our results suggest that exogenous glycine can influence membrane properties, intracellular pH and calcium. The ability of glycinergic terminals to evoke this sort of response remains to be tested.

5.3. A Developmental Shift from GABAergic to Glycinergic Transmission

Having assumed that the MNTB projection was glycinergic from birth, we rarely tested its pharmacology. When we did so, as shown in Fig. 5 (inset), SN was usually able to block most of the MNTB-evoked IPSP. However, during a more detailed voltage clamp analysis, we were surprised to learn that inhibition is primarily GABAergic at first, and only becomes glycinergic during the first two postnatal weeks (Kotak *et al.*, 1998). When voltage clamp recordings were made from LSO neurons at P3–16 in the presence of glutamate receptor antagonists (20 μM CNQX plus 50 μM AP5, or 5 mM kynurenic acid), it was possible to record MNTB-evoked IPSCs in the majority of neurons. Figure 10A shows examples of MNTB-evoked IPSCs at P4 and P14. At P4, most of the IPSC was blocked with the $GABA_A$ receptor antagonist, bicuculline (10 μM). In contrast, the major IPSC component was blocked by SN (2 μM) at P14. This change was highly significant: The bicuculline-sensitive IPSC component declined from 115 pA at P3–5 to 15 pA at P12–16, and there was a complementary increase in the SN-sensitive component (from 30 pA at P3–5 to 125 pA at P12–16). This transition seems to occur only in the medial (high frequency) region of LSO, the target of the majority of MNTB axons (Sanes and Siverls, 1991).

Of course, is remains possible that the MNTB stimulating electrode activates a transient GABAergic projection in young animals, and MNTB neurons do not change phenotype. However, there are other observations that lend to support the hypothesis that

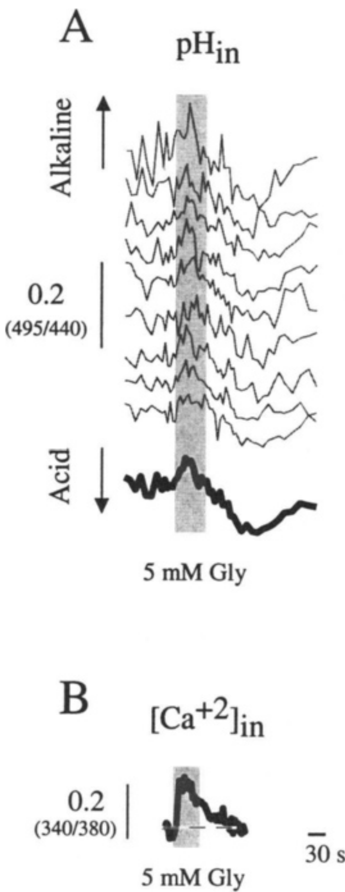

Figure 9. pH and Calcium transients in LSO neurons in response to bath application of 5 mM glycine. (A) Glycine superfusion leads to a transient alkalinization, followed by an acidification. LSO slices were incubated in BCECF, and 9 neurons were monitored during glycine exposure (thin lines). The average trace (thick line) is shown at the bottom. (B) Glycine exposure usually led to a transient increase in intracellular calcium. LSO slices were incubated in Fura-2, and the bold trace shows an average of 8 neurons.

inhibitory projections are at least partially GABAergic during early development. First, when P3–5 LSO neurons are exposed to GABA or glycine at equivalent concentrations, GABA produces a significantly larger and longer lasting change in holding current. Second, there is a reduction in staining for the $\beta_{2,3}$ $GABA_A$ receptor subunit in LSO from P4 to P14, while staining for the glycine receptor-associated protein, gephyrin, increases (Kotak *et al.*, 1998). Third, immunohistochemical staining for GABA in the MNTB of neonatal ferrets (Henkel and Brunso-Bechtold, 1998) and gerbils (Korada and Schwartz, 1999) suggest that it is expressed in neonates.

The early development of GABA-containing neurons is found in many areas of the nervous system (Lauder *et al.*, 1986), and transient GABA expression in spinal neurons is well described (Obata *et al.*, 1978; Reitzel *et al.*, 1979; Maderdrut *et al.*, 1986; Ma *et al.*, 1992; Mitchell and Redburn, 1996). In fact, a developmental decrease in GABA-positive neurons in the chick spinal cord is accompanied by an increase in glycinergic cells, possibly reflecting a change in transmitter phenotype (Berki *et al.*, 1995).

These findings do not necessarily run counter to our previous experimental findings. Contralateral cochlear ablations should have denervated the MNTB, which forms an inhibitory projection, albeit a mixed GABAergic and glycinergic one. SN should also have attenuated the inhibitory synapses from the outset because glycinergic transmission was, in fact, found at all ages examined. What, then, are we to make of the GABAergic

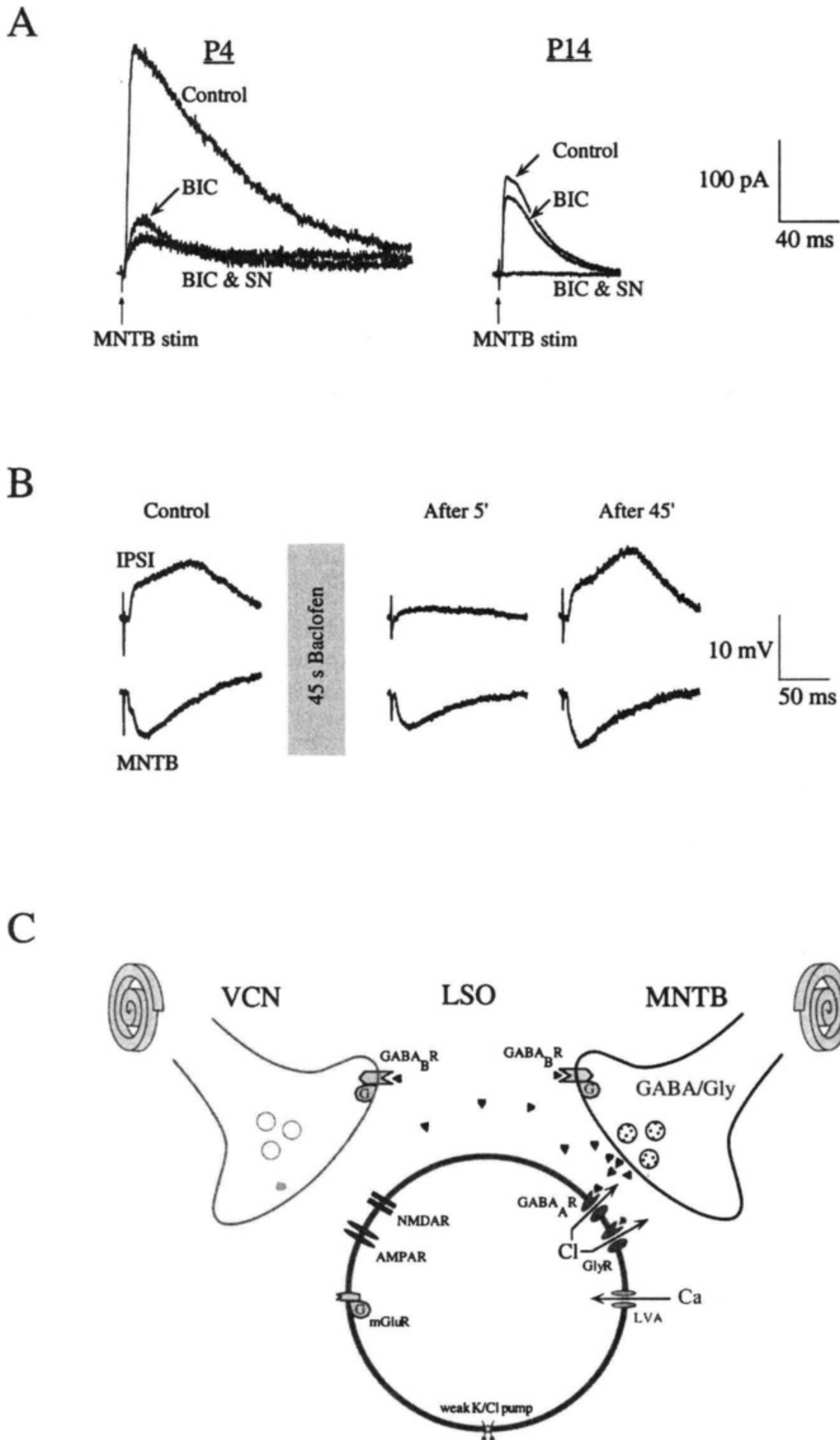

Figure 10. Inhibition is GABAergic in the neonatal LSO. (A) For medial limb neurons, MNTB-evoked IPSCs are primarily BIC-sensitive at P4 (left). In contrast, BIC eliminates only a minor fraction of the IPSC at P14, although SN eliminates the remainder (BIC & SN). (B) Baclofen causes a long-lasting depression of synaptic transmission in the LSO. In current-clamp recording from a P6 medial limb neuron, ipsilaterally-evoked (IPSI) EPSPs and MNTB-evoked IPSPs are depressed following exposure to the $GABA_B$ agonist, baclofen (100 μM). The response recovered fully by 45 mins. The input resistance of this neuron did not change during the recording period (not shown). (C) The hypothetical drawing shows how GABAergic inhibition might allow inhibitory terminals to depress neighboring synapses via presynaptic $GABA_B$ receptors.

inhibition? Is it merely an error in phenotype, or does it serve a purpose during the development of inhibitory connections?

One possibility that occurred to us is that GABA is released in neonates because it can activate a metabotropic pathway, a mechanism that is unknown for glycinergic systems. In fact, pharmacological activation of $GABA_B$ receptors produced an extended depression of ipsilateral excitatory and MNTB-evoked inhibitory synaptic responses (Fig. 10B), although the electrical properties of postsynaptic LSO neurons remained largely unchanged (not shown). The prolonged time course of synaptic depression (10–15 min) suggests that metabotropic $GABA_B$ receptors may be located on afferent terminals (Bowery, 1989; Barthel *et al.*, 1996; Tremblay *et al.*, 1995; Zhang *et al.*, 1997). One speculative model (Fig. 10C) proposes that inhibitory terminals release GABA in order to depress neighboring terminals through a presynaptic mechanism, perhaps enhancing their own ability to form a stable synaptic contact.

While this model may appear to be a remote possibility, it does make the prediction that stimulation of MNTB afferents should lead to long term depression of many or most terminals in the vicinity. We have recently tested this hypothesis by using a stimulus regimen that has been popular with those studying long term depression (LTD) in cortex and hippocampus (1 Hz stimulation for several minutes). As Fig. 11 shows for one LSO neuron, a low rate of stimulation to the MNTB (in the presence of 5 mM kynurenic acid) produced a long-lasting depression of the MNTB-evoked IPSC. Similar findings have been obtained in 21 recordings from P8–12 animals (Kotak and Sanes, 1998). Furthermore, the effect depends on postsynaptic levels of intracellular free calcium: when the calcium chelator, BAPTA, is included in the recording pipet, long term depression of these inhibitory synapses was completely blocked (Kotak and Sanes, 1999). These results suggest the novel hypothesis that inhibitory terminals compete with one another, and perhaps with excitatory terminals, based on a synaptic mechanism of long term depression. Although purely speculation at this point, it is possible that GABAergic transmission is prominant in young neurons because the LTD, and synapse elimination, utilizes metabotropic $GABA_B$ receptors.

6. ACCENTUATE THE NEGATIVE

Most studies of developmental plasticity have focused attention on modifications that may occur at excitatory synaptic contacts. This is entirely reasonable. After all, much of the evidence comes from studies in which single neuron coding properties are assessed by extracellular recording of action potentials in the CNS. While this depends absolutely on the presence of excitatory synaptic input, the contribution of inhibitory synapses is less certain. Furthermore, one of the most informative model systems, the mammalian nerve-muscle junction, is exclusively excitatory, allowing one to ignore the contribution of inhibition.

How, then, do the present studies on inhibitory development fit into the broader picture? The first general conclusion is that inhibitory synaptic activity does contribute to postsynaptic maturation, including dendrogenesis and expression of ionic channels. Perhaps the most interesting aspect of these findings is that inhibitory synaptic activity in the LSO seems to modulate the strength of excitatory terminals by regulating the expression of functional NMDA receptors.

In vitro experiments have previously implicated GABAergic signalling in process outgrowth (Michler-Stuke and Wolff, 1987; Mattson and Kater, 1989; Spoerri, 1988;

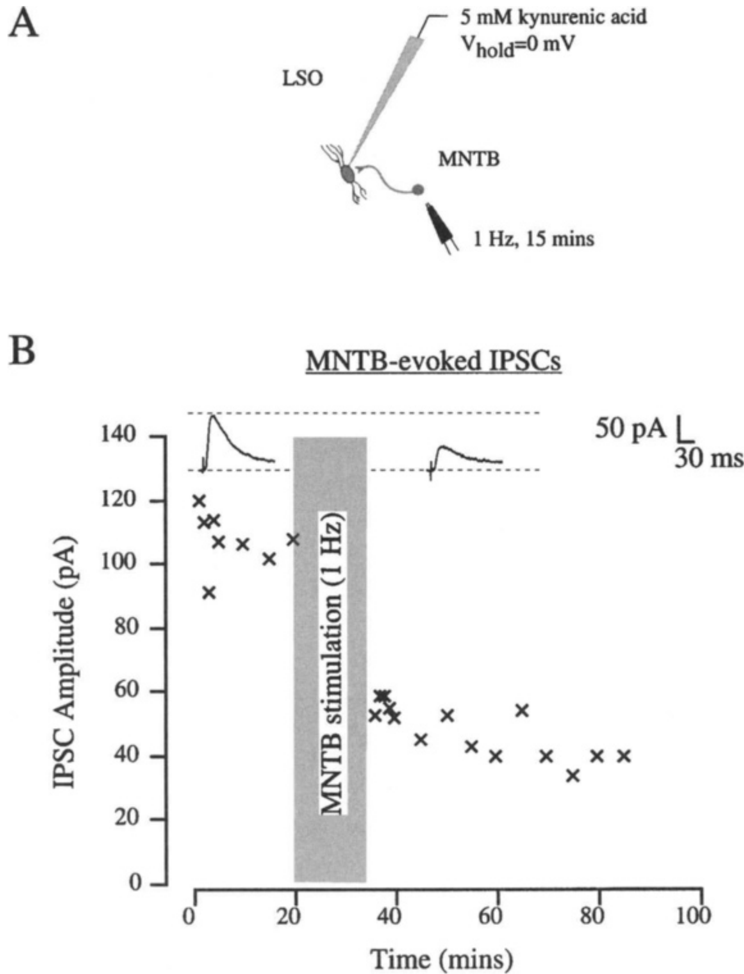

Figure 11. Long term synaptic depression of inhibitory synapses in the developing LSO. (A) A whole cell voltage clamp recording ($V_{hold} = 0\,mV$) was obtained in a P10 neuron in the presence of 5 mM kynurenic acid (to block all ionotropic glutamatergic synapses). MNTB-evoked IPSCs were recorded for 20 mins to establish a baseline, and 1 Hz stimulation was then delivered to the MNTB pathway for 15 mins. (B) The stimulated synapses were depressed by the treatment, and remained so for the duration of the recording (up to 4 hours). Nearly identical observations were made in 21 other LSO neurons using the same paradigm.

Behar *et al.*, 1996), synaptogenesis (Redburn, 1992; Corner and Ramakers, 1992), and $GABA_A$ receptor expression (Frieder and Grimm, 1985; Liu *et al.*, 1997; Hablitz *et al.*, 1989; Montpied *et al.*, 1991; Kim *et al.*, 1993; Barbin *et al.*, 1993; Behar *et al.*, 1994; Poulter *et al.*, 1997). The present studies extend this concept to glycinergic signalling, and employ two *in vivo* manipulations to show that inhibitory transmission is operational during normal development.

A second general conclusion is that inhibitory synaptic contacts appear to be quite dynamic during development. Our descriptive data indicate that IPSP duration decreases by an order of magnitude, functional inhibitory synapses are eliminated, and glycine receptor expression is modified. Our experimental data suggests that disuse can result in

smaller IPSPs and inhibitory arbors that do not go through the process of anatomical refinement.

Disuse of inhibitory synapses could affect both presynaptic transmitter release and postsynaptic transduction. The present results show that MNTB-evoked IPSPs are weaker following disuse, suggesting that the presynaptic terminal is no longer releasing transmitter. In this regard, it is interesting that conjoint recordings from pre- and postsynaptic neurons in the goldfish brain stem have documented "silent" glycinergic synapses. These synapses can be activated following tetanic stimulation of the presynaptic cell (Charpier *et al.*, 1995). Our result also raise the possibility the chloride equilibrium potential has depolarized following disuse, suggesting that a transport mechanism may be regulated by inhibitory activity (Rivera *et al.*, 1997).

A third general conclusion is that different transduction pathways should be considered when trying to explain the development and plasticity of inhibitory synapses. Whereas some traditional mechanisms may apply, the present results raise a few intriguing alternatives.

Although any number of cellular mechanisms might allow inhibitory terminals to influence neuronal maturation, much attention has been focused on the depolarizing IPSPs that are found in neonatal animals. In adult animals, synaptic inhibitory potential are generally hyperpolarizing because the receptor is coupled to a Cl^- channel, and the Cl^- equilibrium potential is more negative than the cells resting potential. However, depolarizing IPSPs are commonly found in early development (Obata *et al.*, 1978; Mueller *et al.*, 1983, 1984; Ben-Ari *et al.*, 1989; Swann *et al.*, 1989; Zhang *et al.*, 1991; Wu *et al.*, 1992; Reichling *et al.*, 1994; Wang *et al.*, 1994; Kandler and Friauf, 1995; Boehm *et al.*, 1997). Synaptic inhibition may also produce postsynaptic depolarizations in an indirect fashion. In the LSO, hyperpolarizing IPSPs may be large enough to recruit a rebound depolarization (Sanes, 1993), possibly due to the presence of a low voltage activated Ca^{2+} channel (Llinás and Yarom, 1981). Since depolarizing IPSPs are able to recruit voltage-gated calcium channels or NMDA-coupled channels, they can raise intracellular free calcium (Connor *et al.*, 1987; Obrietan and van den Pol, 1995; Ben-Ari *et al.*, 1997). It is also reasonable to assume that synaptic inhibition can introduce a current shunt, thus preventing the calcium influx that would normally accompany excitatory synaptic activity (Callaway *et al.*, 1995; Lo *et al.*, 1998).

If inhibitory synapses do modulate calcium during development, they may take advantage of mechanisms used by excitatory synapses (Connold *et al.*, 1986; Wang *et al.*, 1994; Lo and Poo, 1994; Zou and Cline, 1996). For example, heterosynaptic depression can be produced in nerve-muscle cultures by stimulating one of the presynaptic neurons (Lo and Poo, 1991). Furthermore, it is possible to cause synaptic depression by momentarily raising postsynaptic calcium in the postsynaptic muscle cell (Cash *et al.*, 1996).

An alternative mechanism by which inhibitory synapses could regulate postsynaptic neurons is through a bicarbonate conductance. Glycine and $GABA_A$ receptor-coupled channels are permeable to HCO_3^- ions (Bormann *et al.*, 1987), and the HCO_3^- equilibrium potential (E_{HCO3}) is more positive than the chloride equilibrium potential (E_{Cl}). When crustacean muscle fibers are bathed in a Cl^--free, bicarbonate-containing solution, E_{GABA} is approximately equal to E_{HCO3} (Kaila *et al.*, 1989). Therefore, a transient HCO_3^- conductance may effect both membrane potential and pH (Kaila and Voipio, 1987; Kaila *et al.*, 1990b; Kaila *et al.*, 1992; Kaila *et al.*, 1993; Staley *et al.*, 1995). To date, there is no direct evidence implicating a pH mechanism during inhibitory plasticity.

Our most recent data raise an intriguing new possibility. It is apparent that synaptic inhibition is GABAergic, at first, particularly in the medial limb of the LSO. One

might wonder why the nervous system would trade in one perfectly reasonable set of ligand-gated chloride channels for another. One possibility is that the GABA provides a signalling pathways that is unavailable to glycine. In fact, glycine receptors appear to be solely ionotropic, whereas baclofen-sensitive $GABA_B$ mechanisms can modulate second messenger pathways (Barthel *et al.*, 1996; Tremblay *et al.*, 1995; Zhang *et al.*, 1997).

Perhaps most critical for the LSO circuit is that equivalent numbers of excitatory and inhibitory synapses are made on each postsynaptic neuron during development, permitting the cells to compute interaural level differences over a reasonably broad range of absolute sound level. This would seem to require a mechanism whereby inhibitory terminals can compete with excitatory terminals for postsynaptic space, and the present studies may provide some insight into this mechanism. Thus, inhibitory terminals may release GABA during the earliest phase of synaptogenesis in order to depress neighboring terminals, presynaptically, possibility increasing the likelihood that these inactive terminals will be eliminated (Fig. 10C). In this regard, it is interesting that a metabotropic glutamatergic system is also active in LSO, and recent data suggests that it, too, may participate in long term depression (Kotak and Sanes, 1995; Kotak and Sanes, 1998).

Sensory stimulation patterns that influence brain development (cf, Fig. 1) can usually be translated in a pattern of EPSPs and action potentials somewhere in the brain, and activity-dependent modification of excitatory synapses plays a key role in neural plasticity. In contrast, we are just beginning to understand that inhibitory synaptic activity can also influence brain development. Since most, if not all, brain circuits receive inhibitory projections, it will be fascinating to learn how these contacts are modified by use, how they interact with excitatory synapses during development, and how their modification effects animal behavior.

7. ACKNOWLEDGMENTS

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8

METAMORPHOSIS AS MIDLIFE CRISIS

What to Do with the Leftover Neurons?

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The midlife crisis occurs in virtually all males, including members of the animal kingdom. A good example is the caterpillar. He will spend a large part of his life on a predictable career path, engaging in traditional caterpillar activities such as crawling around and munching on leaves of expensive ornamental shrubbery, and then one day, out of the blue, he'll say to his wife, "Dammit, Louise, I'm sick of shrubbery." She does not understand him, of course. Partly this is because she has a brain the size of an electron, but mostly it is because he seems like a total stranger to her, a different insect altogether. Soon he has left her to live in his own cocoon, from which he eventually emerges with a whole new "youthful" look—wings, bright colors, gold jewelry, etc. As he soars into the sky, feeling fulfilled and exhilarated, free at last from the restrictive routines of his humdrum former life, Louise watches him from far below. She feels conflicting emotions: sorrow, for she knows that she has lost her mate forever; but also a strange kind of joy, for she also knows, as she watches his multihued wings flashing in the glorious golden-red glow of the sinking sun, that he is about to be eaten by a bat.

DAVE BARRY, 1990

1. INTRODUCTION

The above quotation from the book, *Dave Barry Turns 40*, is an appropriate opening to this chapter for two reasons. First, the conference on "The Biology of Early Influences", which led to the book of the same name, was held in Florida, the base of operations for Dave Barry's syndicated column in the *Miami Herald.* Second, Barry's theme of "metamorphosis as midlife crisis" is a perfect segue into the topic of this chapter: what to do with leftover neurons when it's time to move on to the next life stage? At the onset of metamorphosis, as caterpillars abandon their humble, earthbound existence to begin the transformation to a life of flight and sexual reproduction, the nervous system is left with a certain number of functionally obsolete neurons. As will be discussed below, two potential fates for such neurons are respecification for new functions or elimination by programmed cell death (PCD).

The Biology of Early Influences, edited by Hyson and Johnson
Kluwer Academic / Plenum Publishers, New York, 1999.

The disposition of functionally obsolete neurons is just one of the many challenges facing the nervous systems of insects and amphibians during metamorphosis (see reviews in Gilbert *et al.*, 1996). My laboratory has investigated these issues using the hawkmoth, *Manduca sexta*, which undergoes metamorphosis from a larva to a pupa to an adult moth in approximately 6 weeks. In *Manduca*, individually identified neurons can be tracked throughout the lifespan, and intrinsic and extrinsic signals that regulate neuronal fate can be identified and manipulated. Furthermore, the same neurons that are studied *in vivo* can be removed from the central nervous system (CNS) and placed in primary cell culture, allowing their autonomous developmental capabilities to be examined in isolation. As discussed below, the metamorphic phenomena that we study, including dendritic remodeling and PCD, are controlled by a small ensemble of hormones that includes juvenile hormone (JH) and the ecdysteroids, a class of steroid hormone. Natural or induced changes in hormone levels can be directly linked to changes in neuronal phenotype, making *Manduca* an ideal system in which to investigate how hormones regulate neuronal structure and function. The other major insect model for studying metamorphosis is the fruitfly, *Drosophila melanogaster. Drosophila* and *Manduca* offer complementary advantages and there is increasing convergence of research on these two species (e.g., reviews by Levine *et al.*, 1995; Truman, 1996).

This review focuses on ecdysteroid effects on *Manduca* neurons, which are mediated via intracellularly-located ecdysteroid receptors (EcRs). These ligand-gated transcription factors belong to the same nuclear hormone receptor superfamily as do receptors for estrogens, androgens, glucocorticoids and non-steroidal lipophilic hormones such as thyroid hormone (reviewed by Mangelsdorf *et al.*, 1995; Thummel, 1995). The evolutionary conservation of receptor structure, and the parallels between steroid action on insect and vertebrate neurons (reviewed by Weeks and McEwen, 1997), suggest that underlying mechanisms may be shared. Likewise, PCD—an active program of cellular suicide—also shows a high degree of evolutionary conservation across species (reviewed by White and Steller, 1995; Ameisen, 1996; Burek and Oppenheim, 1996). Thus, insights into the steroid regulation of neuronal phenotype in insect systems are relevant to understanding nervous system development and plasticity in other species.

Recent reviews have discussed phenotypic changes in *Manduca* neurons during metamorphosis and their endocrine control (Weeks and Levine, 1992), hormonal effects on the electrophysiological function of neural circuits for behavior (Weeks *et al.*, 1997), comparisons of neural plasticity during metamorphosis and learning (Weeks and Wood, 1996), the utility of combined *in vivo* and *in vitro* approaches (Levine and Weeks, 1996) and comparisons between *Manduca* and vertebrate systems for investigating steroid hormone effects on neurons and behavior (Weeks and Levine, 1995; Weeks and McEwen, 1997). The present review focuses on the fates of functionally obsolete neurons during *Manduca* metamorphosis and, in particular, extrinsic and intrinsic determinants that regulate dissimilar metamorphic fates of segmentally-repeated larval neurons.

2. *MANDUCA* PROLEG MOTONEURONS UNDERGO RESPECIFICATION OR DEATH AT THE END OF LARVAL LIFE

This review focuses on a segmentally-repeated complement of abdominal motoneurons in *Manduca* that exhibit segment-specific fates during metamorphosis (Fig. 1, Table 1). These motoneurons are designated accessory planta retractors (APRs), based on their

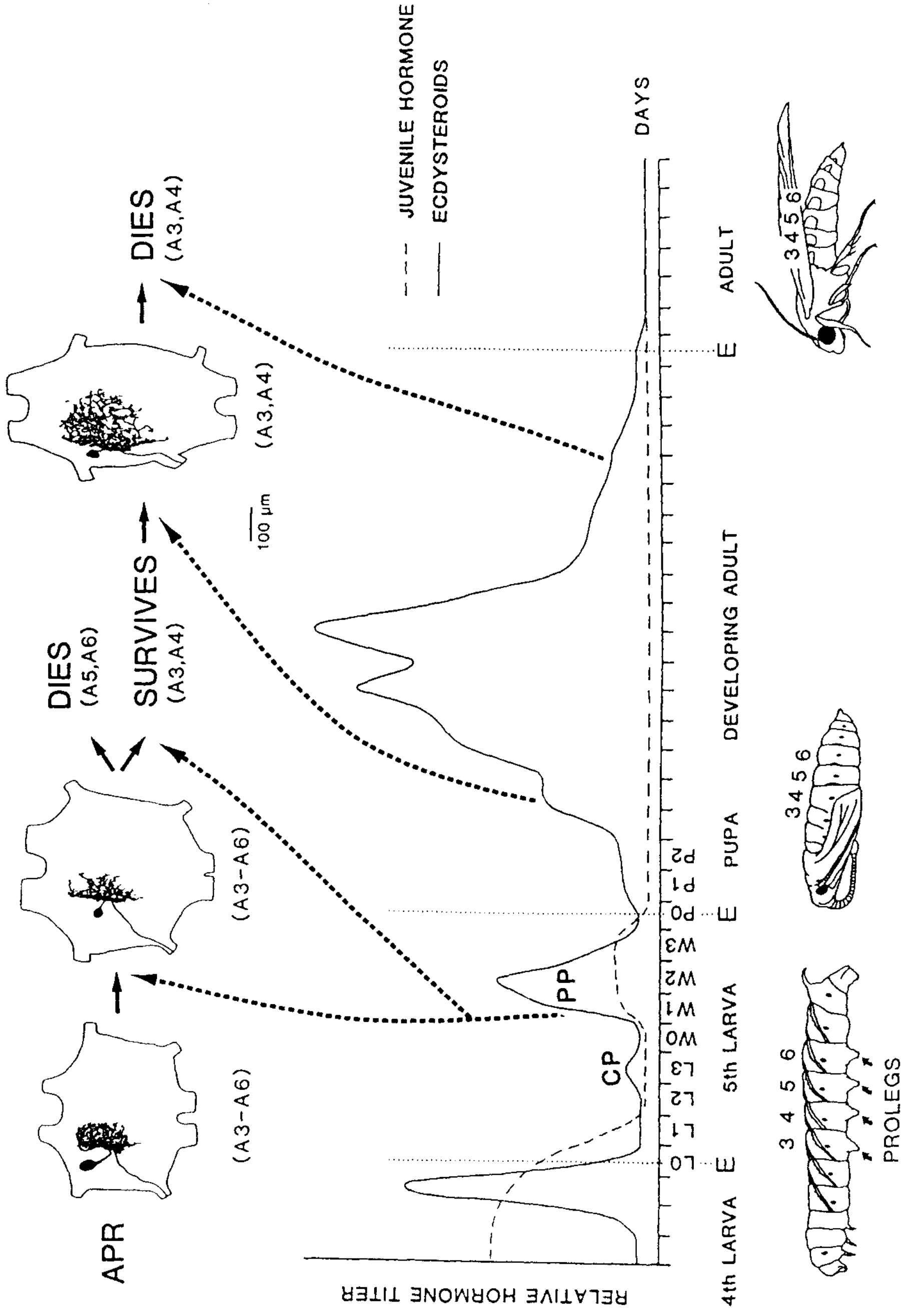
Table 1. Segment-specific fates of APRs and APRMs at pupation[1]

Segment	Motoneuron (APR) fate at pupation	Muscle (APRM) fate at pupation	Pupal function
A1	Dies	Dies	
A2	Lives	Lives	Hemolymph circulation in developing wings/legs
A3	Lives	Lives	Hemolymph circulation in developing wings/legs
A4	Lives	Dies	APRs innervate new abdominal extensor muscles
A5	Dies	Dies	
A6	Dies	Dies	

[1]From Weeks and Ernst-Utzschneider (1989), Sandstrom and Weeks (1998), and Lubischer *et al.* (1999).

innervation of accessory planta retractor muscles (APRMs) in larval abdominal ganglia three through six (A3–A6) (Fig. 2A; Weeks and Truman, 1984; Sandstrom and Weeks, 1996). APRMs are retractor muscles of the prolegs, which are the primary locomotory appendages of larvae (caterpillars). There is one APRM per proleg and contraction of this muscle withdraws the proleg toward the body. Homologs of the APRs and APRMs are also present in non-proleg-bearing larval segments A1 and A2 (Fig. 2A), where they presumably contribute to abdominal tonus and bending movements (Weeks and Ernst-Utzschneider, 1989). APRMs and APRs are designated by their segmental location [e.g., APR(3)s are the APRs located in ganglion A3]. Throughout larval life (~2.5 weeks under laboratory conditions), APRMs and APRs participate in various proleg behaviors, which include clinging to plants during feeding, crawling, pre-ecdysis (cuticle-loosening) and ecdysis (cuticle-shedding) behaviors, and local reflexes evoked by sensory stimulation (Copenhaver and Truman, 1982; Weeks and Truman, 1984; Weeks and Jacobs, 1987; Miles and Weeks, 1991; Novicki and Weeks, 1993; Johnston and Levine, 1996; Wiel and Weeks, 1996; Wood *et al.*, 1997).

Although the APRs and APRMs play critical behavioral roles during larval life, their utility is abruptly cut short at pupation when the prolegs degenerate. Unlike larval legs in the thorax, which are later replaced by new adult legs (Kent and Levine, 1988), the abdominal prolegs are not replaced. What, then, is the fate of the functionally obsolete APRMs and APRs? The answer to this question is that, in a complex segment-specific pattern, APRMs and APRs are either eliminated by PCD or retooled to serve entirely new functions during pupal life. Figure 1 illustrates phenotypic changes in APRs during these events. During the final days of larval life, the dendrites of APRs in all segments undergo a period of dramatic regression, which is associated with the loss of synaptic inputs involved in larval behaviors (Streichert and Weeks, 1995). In this way, the APRs are extricated from neural circuits for larval behavior. All APRs are alive on the first day of pupal life (day P0) but, within the next 24 to 48 hours, a segment-specific pattern of PCD eliminates the APR(1)s, APR(5)s and APR(6)s (Table 1) (Weeks and Ernst-Utzschneider, 1989; Weeks *et al.*, 1992). Many other abdominal motoneurons also die at this time, reflecting the progressive simplification of the abdominal musculature during metamorphosis (Levine and Truman, 1985). Interestingly, APRMs also undergo segment-specific PCD at pupation, but in a pattern different than that of the APRs: APRMs degenerate in all segments except A2 and A3 (Fig. 2A; Sandstrom and Weeks, 1998). As shown in Table 1, the outcome of these events produces three patterns of muscle and motoneuron fate. In segments A1, A5 and A6, both the muscles and motoneurons die. Thus, the APRs and APRMs in these segments play a role only during larval life and are then eliminated. In segment A4, the muscles degenerate but the motoneurons survive; the APR(4)s are respecified to innervate new muscles (abdominal extensors) that develop

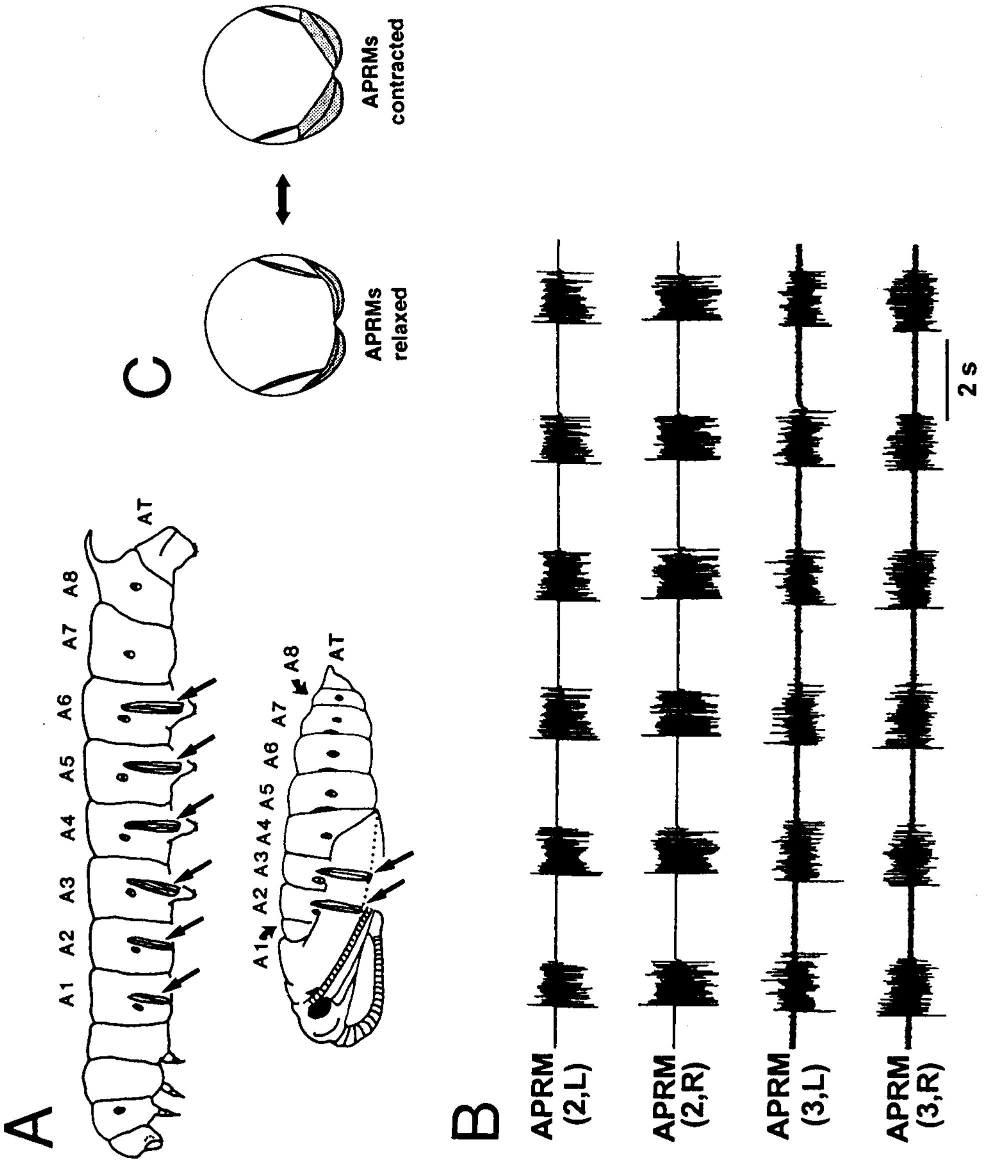

APR
(A3–A6)
(A3–A6)
DIES
(A5,A6)
SURVIVES
(A3,A4)
(A3,A4)
DIES
(A3,A4)
100 µm
JUVENILE HORMONE
ECDYSTEROIDS
DAYS
CP
PP
RELATIVE HORMONE TITER
L0
L1
L2
L3
W0
W1
W2
W3
P0
P1
P2
E
E
E
4th LARVA
5th LARVA
PUPA
DEVELOPING ADULT
ADULT
3 4 5 6
PROLEGS
3 4 5 6
3 4 5 6

during the pupal stage (Weeks and Ernst-Utzschneider, 1989). This type of motoneuron respecification has been well characterized by others (e.g., Levine and Truman, 1985; Truman and Reiss, 1995) so was not considered further. Instead, we focused on a novel type of respecification that occurs in segments A2 and A3, in which both APRs and APRMs survive (Fig. 2A).

The APRMs and APRs in segments A2 and A3 are respecified in the following way. During larval life, the mass of individual APRM(3)s is approximately three times that of the APRM(2)s. At pupation, APRMs in both segments lose mass such that all four muscles stabilize at the same reduced mass for the remainder of the pupal stage. Thus, the larval muscles converge in size and function early in the pupal stage. The function of the retained APRM(2)s and APRM(3)s was investigated by electromyographic (EMG) recordings, which revealed a robust, rhythmic motor pattern in which all four APRMs contract at approximately 5 s intervals for bouts of hundreds of cycles (Fig. 2B; Sandstrom and Weeks, 1998). The APRM contractions are driven by rhythmic synaptic depolarizations of APRs, in a centrally generated rhythm we have termed the "pupal motor pattern". The anatomical location of APRM insertion points in the pupal body, and simultaneous EMG and thermographic recordings of hemolymph (blood) flow in intact pupae, revealed that the pupal motor pattern circulates hemolymph within the lumina of the developing adult wings and legs (Fig. 2C; Sandstrom and Weeks, 1998; Lubischer *et al.*, 1999). This represents, to our knowledge, the most extreme functional respecification of a neuromuscular system reported in *Manduca*; motoneurons and muscles that participate in abdominal bending movements or proleg retractions in larvae are retooled for a circulatory function in pupae. The appearance of this new motor pattern at pupation must involve changes in synaptic inputs to the APR(2)s and APR(3)s, at a time when these motoneurons are maximally regressed (Fig. 1) and are losing synaptic inputs for larval behaviors (Streichert and Weeks, 1995). How these synaptic changes occur in the context of ongoing dendritic loss remains to be investigated. Interneurons that make monosynaptic connections with larval APRs and could potentially participate in the pupal motor pattern have been identified (Sandstrom and Weeks, 1991).

The APRs that survive through the pupal stage in segments A2, A3 and A4 undergo dendritic regrowth such that their arbors in pharate adults (fully-formed adult moths still encased in the pupal cuticle) are more extensive and have a different shape than those in larvae (Fig. 1) (Weeks and Ernst-Utzschneider, 1989). The relationship between dendritic growth and the reorganization of synaptic inputs to respecified APRs is unknown, but

Figure 1. Metamorphic changes in *Manduca* hormones and APRs. Camera lucida drawings across the top show phenotypic changes in APRs during metamorphosis (the outlines of abdominal ganglia are shown; anterior is up), with the relevant abdominal segments indicated in parentheses. The plot below shows the relative hemolymph (blood) titers of ecdysteroids and juvenile hormone (JH) during metamorphosis. Days during the larval-pupal transformation are labeled. Specific changes in hemolymph levels of ecdysteroids and JH regulate phenotypic changes in APRs (linked by dashed arrows). The prepupal peak of ecdysteroids triggers regression of APRs in all segments and death of APRs in a subset of segments. Regrowth of respecified APRs is triggered by the pupal rise in ecdysteroids and the death of APRs after emergence results from the preceding fall in ecdysteroids. Drawings at the bottom show larval, pupal and adult *Manduca*, with abdominal segments A3–A6 labeled and the larval prolegs in A3 to A6 indicated by arrows. Abbreviations: E, ecdysis (the shedding of the old cuticle); CP, commitment pulse; L0, the day of entry into the 5th larval instar; P0, the first day of pupal life [pupal ecdysis (PE) occurs on this day]; PP, prepupal peak; W0, wandering. Hormone titers modified from Bollenbacher *et al.* (1981) and Riddiford and Truman (1994). Figure reprinted from Weeks and McEwen (1997), by permission of The MIT Press.


A
A1
A2
A3
A4
A5
A6
A7
A8
AT
B
APRM (2,L)
APRM (2,R)
APRM (3,L)
APRM (3,R)
2 s
C
APRMs relaxed
APRMs contracted

has been investigated for other *Manduca* motoneurons (Levine and Truman, 1982). An interesting observation is that the pupal motor pattern continues to be expressed throughout the pupal stage (Sandstrom and Weeks, 1998), suggesting that electrophysiological properties of synaptic inputs to APRs that drive activity during the pattern are maintained despite ongoing dendritic remodeling and electrotonic changes in the motoneurons. Adult emergence is followed by another wave of motoneuron death, as the abdominal musculature is simplified even further (Truman, 1983; Levine and Truman, 1985). The remaining APRs undergo PCD at this time (Fig. 1), as do the APRM(2)s and APRM(3)s; circulation of hemolymph within the adult wings and legs is taken over by accessory pulsatile organs that develop within the thorax during the pupal stage (Krenn and Pass, 1994). Thus, the last remnants of this larval neuromuscular system are eliminated early in the adult stage. Other muscles and motoneurons generate abdominal movements of the adult moth.

3. HORMONAL CONTROL OF PHENOTYPIC CHANGES IN *MANDUCA* PROLEG MOTONEURONS DURING METAMORPHOSIS

3.1. Regulation of Phenotypic Changes in APRs by Ecdysteroids and JH

In *Manduca*, the "white rat of insect endocrinology", we are fortunate to know a great deal about one class of extrinsic signals that regulate neuronal phenotype—the hormones that drive metamorphosis (see Nijhout, 1994; Gilbert *et al.*, 1996). Figure 1 shows hemolymph levels of JH and ecdysteroids from the penultimate larval instar (stage) through adult emergence, and the relationship between hormonal events and phenotypic changes in APRs. Each molt is triggered by an elevation of ecdysteroids, with the type of molt (i.e., larval-larval, larval-pupal, or pupal-adult) determined by the JH level and the previous history of hormonal exposure. Molts from one larval instar to the next are triggered by a surge of ecdysteroids in the presence of JH. In the final larval instar, meta-

Figure 2. Respecification of larval APRs and APRMs in segments A2 and A3 for a circulatory function in pupae. A. Drawings of a larva (top) and pupa (bottom), with anterior to the left and abdominal segments 1 to 8 (A1 to A8) and the terminal abdominal segment (AT) indicated. In larvae, APRM homologs (arrows) are present in proleg-bearing segments A3 to A6 and non-proleg-bearing segments A1 and A2. In pupae, the retained APRMs in segments A2 and A3 (arrows) insert ventrally on the flexible abdominal floor (dotted line), beneath the developing wings and thoracic legs that extend posteriorly over the ventral abdomen. B. Electromyographic (EMG) recording of the pupal motor pattern in an intact pupa. Simultaneous EMG recordings from the four APRMs in an intact day P3 pupa are shown. The abdominal segment (2, 3, etc.) and body side (R, right; L, left) of the recording sites are indicated. The pupal motor pattern consists of rhythmic, synchronous contractions in all four APRMs at approximately 5 s intervals. C. Hypothesized role of pupal APRMs in hemolymph circulation. A transverse view of segment A3 is illustrated (dorsal up), with the two APRMs shown relaxed (left) or contracted (right). The ventral surface of segment A3 is covered by the developing metathoracic wings, which are in turn covered by the developing mesothoracic wings. The lumina of the wings are shown stippled. The segment is bounded entirely by rigid cuticle while the ventral abdominal surface and adjacent wing surfaces are flexible. When the APRMs are relaxed, the wings lie in their resting positions. APRM contraction pulls the wings dorsally, increasing the volume of their lumina and pulling hemolymph into the lumina from their openings to the thorax. The action is similar to that of a bellows. In segment A2 (not shown), APRMs insert over the metathoracic legs, which are covered by the meso- and metathoracic wings. Figure modified from Sandstrom and Weeks (1998), by permission of The Company of Biologists, Ltd.

morphosis is initiated by a small pulse of ecdysteroids in the absence of JH (the *commitment pulse*) which commits cells to subsequent pupal development and triggers wandering behavior, when the larva burrows underground to construct a pupation chamber. The larval-pupal molt is then triggered by a *prepupal peak* of ecdysteroids in the presence of a low level of JH. Following pupation, development of the adult moth is driven by a prolonged rise and fall of ecdysteroids in the absence of JH. Male and female *Manduca* have similar hormone titers during metamorphosis (Baker *et al.*, 1987).

The relationship between developmental changes in ecdysteroid and JH levels, and phenotypic changes in proleg motoneurons, was determined by extirpation and replacement experiments; i.e., the elimination of endocrine glands and replacement with exogenously supplied hormones (Weeks and Truman, 1985, 1986; Weeks, 1987; Weeks and Ernst-Utzschneider, 1989; Weeks *et al.*, 1992, 1993; Zee and Weeks, 1998 and in preparation). More recently, *in vivo* experiments have been complemented by cell culture experiments (see Section 4). As indicated in Fig. 1, the regression and PCD of proleg motoneurons at pupation is triggered by the rising phase of the prepupal peak of ecdysteroids; JH present during the prepupal peak has no influence on these events. However, appropriate responses to the prepupal peak of ecdysteroids depend critically on the prior *absence* of JH during the commitment pulse; if JH is present (e.g., due to exogenous application), motoneurons retain the larval *status quo* and fail to express pupal phenotypes at the next molt. Dendritic regrowth in proleg motoneurons during the pupal stage is triggered by the elevation of ecdysteroids after pupation, and the death of APRs following adult emergence is triggered by the fall in ecdysteroids at the end of adult development. We have not investigated the endocrine cues for APRM degeneration but, based on studies of other muscles, it is likely that the APRMs die in response to the same signals that trigger APR death (Schwartz and Truman, 1983; Weeks and Truman, 1985, 1986). That APRM degeneration may be a direct response to ecdysteroids is suggested by the finding that denervation has no effect on the degeneration of another proleg muscle at pupation (Weeks and Truman, 1985). Similarly, ablation of target muscles does not perturb the dendritic regression or PCD of proleg motoneurons (see Section 3.3; Weeks and Truman, 1985; Lubischer and Weeks, 1996). A direct action of ecdysteroids on APRs to induce PCD has now been demonstrated in cell culture (Section 4).

In summary, over the *Manduca* lifespan, segmentally-repeated APRs and APRMs exhibit stereotyped, segment-specific fates at different developmental times in response to different hormonal cues. Ecdysteroids and JH are essential extrinsic cues for these developmental changes. The regulation of APRM fate will not be considered further here and we will instead focus on the APRs. Several aspects of the life history of the APRs raise interesting questions. For example, what are the mechanisms by which the prepupal peak of ecdysteroids causes APRs to regress and lose synaptic inputs similarly in all segments (Streichert and Weeks, 1995; Sandstrom and Weeks, 1998) yet produces segment-specific PCD among the same APRs? Also, APRs that die at pupation do so in response to a *rise* in ecdysteroids whereas those that die after emergence do so in response to a *fall* in ecdysteroids (see below); how do segmental location and developmental stage of an APR determine the hormonal stimulus that is required to trigger PCD? Identifying the molecular basis for phenomena such as these remains an important objective for the future.

3.2. Ecdysteroid Receptor Isoforms Are not Correlated with APR Fate

One mechanism that could mediate segment- and/or stage-specific responses to ecdysteroids is the number or type of ecdysteroid receptors (EcRs) expressed in APRs.

Autoradiographic studies show that most, if not all, *Manduca* motoneurons have nuclear EcRs during the prepupal peak (Fahrbach, 1992). Jindra *et al.* (1996) showed that the *Manduca EcR* gene encodes two isoforms, EcR-A and EcR-B1, and the presence of different EcR isoforms is correlated with different cell fates in *Drosophila* and *Manduca* (reviewed by Truman, 1996). We tested the possibility that the segment-specific pattern of APR death at pupation was correlated with differences in EcR expression by examining EcR immunoreactivity in APR(4)s and APR(6)s (which are fated to live and die, respectively, at pupation) using antibodies specific to either or both *Manduca* EcR isoforms (generously provided by Dr. Lynn M. Riddiford, Univ. Washington). Using laser scanning confocal microscopy, we did not detect segmental differences in EcR isoform immunoreactivity in APR(4)s *versus* APR(6)s during the prepupal peak (G. A. Kinch, J. Ewer and J. C. Weeks, unpublished data). It is possible that small but biologically significant segmental differences in EcR isoform levels were present but undetectable by our methods. Alternatively, these data suggest that the segment-specific responses of APRs to ecdysteroids are regulated by events downstream of the binding of ecdysteroids to EcRs (reviewed by Thummel, 1995; Beato *et al.*, 1996).

3.3. Cellular Interactions Do not Regulate Segment-Specific PCD of APRs

Another possible source of cues for segment-specific fates of APRs is extrinsic signals from pre- or postsynaptic neurons, glia, extracellular matrix or other components. This possibility was tested by surgically interrupting APRs' interactions with (1) APRMs, (2) interganglionic interneurons or (3) a major class of mechanosensory neurons that monosynaptically excite APRs. All manipulations were performed early in the final larval instar and APR death was examined at pupation and after adult emergence. None of the perturbations disrupted the segment-specific pattern of APR death at either developmental stage, the only effect being slightly increased variability in the timing of death (Weeks and Davidson, 1994; Lubischer and Weeks, 1996). Thus, no existing evidence supports the possibility that cellular interactions regulate the segment-specific pattern of APR death *in vivo.* These findings led to the alternative hypothesis that APRs posses intrinsic segmental identities that determine their responses to hormones independently of cellular interactions. Experimental support for this hypothesis is described in Section 4.

4. INTRINSIC DETERMINANTS OF SEGMENT-SPECIFIC PCD OF APRS STUDIED IN CELL CULTURE

4.1. Cell Culture Experiments Demonstrate that APRs Possess Intrinsic Segmental Identities that Determine Their Response to Ecdysteroids

To test the hypothesis that the segment-specific PCD of APRs is a cell-autonomous response to ecdysteroids, we removed APRs from the CNS and exposed them to ecdysteroids *in vitro*. Techniques were modified from those used by Dr. Richard B. Levine and collaborators at University of Arizona to culture other *Manduca* motoneurons (Prugh *et al.*, 1992). In an initial study (Streichert *et al.*, 1997), APR(4)s and APR(6)s were retrogradely labeled *in situ* with the vital fluorescent marker DiI, then enzymatically dissociated from ganglia and placed in low-density primary cell culture in hormone-free medium on day W0. Day W0 is immediately prior to the prepupal peak of ecdysteroids (Fig. 1), when APRs are committed to pupal fates and poised to respond to the prepu-

pal peak of ecdysteroids by expressing pupal phenotypes. Each culture dish contained one DiI-labeled APR plus additional unidentified neurons (to serve as positive controls for APR death; see below). When placed in culture, APRs consisted of an isolated soma that sometimes bore a short length of primary neurite. After APRs adhered to the dish and began process outgrowth, they were treated for the remainder of the culture period with hormone-free medium or medium containing 1 or 10 μg/ml 20-hydroxyecdysone (20-HE). These concentrations of 20-HE bracket endogenous levels during the prepupal peak (Bollenbacher *et al.*, 1981). The APRs were observed for 20 days and scored blindly as live or dead from photographs. Live APRs are recognizable by their large, phase-bright somata and intact processes (Fig. 3). APRs were scored as dead when (1) the processes fragmented and (2) the soma rounded up or fragmented (Fig. 3C). These simple morphological criteria for PCD were confirmed by other staining methods (see Section 4.2) and the specificity of APR death was confirmed by the presence of other live neurons in each dish.

The key finding was that APRs continue to express the correct segment-specific pattern of PCD when exposed to 20-HE *in vitro*. Figure 3 presents representative examples of individual APRs. Nearly all APR(4)s survived for at least 20 days, regardless of whether or not 20-HE was present in the medium (Fig. 3A). This result mirrors events

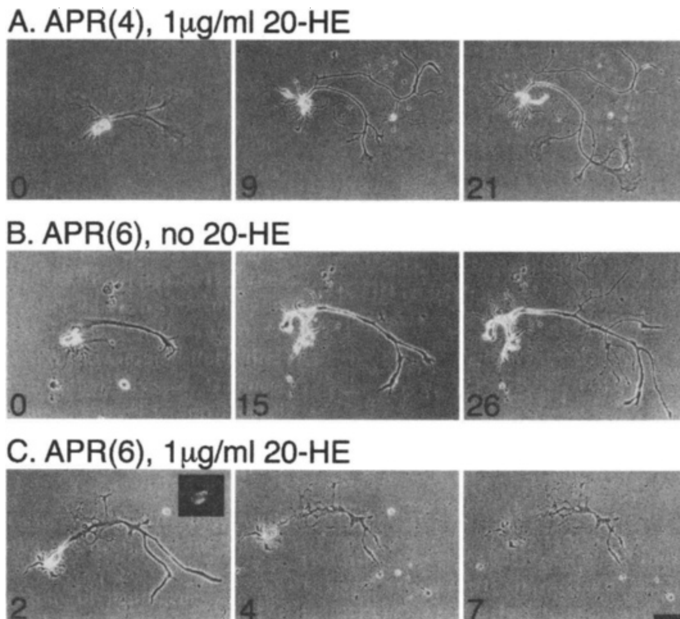


Figure 3. Segment-specific PCD of APRs cultured at wandering, in response to ecdysteroid exposure *in vitro*. DiI-labeled APRs were cultured in hormone-free medium on day W0, immediately prior to the start of the prepupal peak of ecdysteroids (see Fig. 1). After APRs adhered to the dish and extended processes, they were treated for the remainder of the culture period with either medium containing 20-HE or hormone-free medium. Photomicrographic series show APRs from the indicated segments and hormonal conditions, with the number of days after adding 20-HE or hormone-free medium shown. An APR(4) treated with 1 μg/ml 20-HE (A) or APR(6) cultured without 20-HE (B) survived for more than 20 d. An APR(6) treated with 1 μg/ml 20-HE (inset shows DiI labeling of the same neuron) was dead by day 7, as indicated by fragmented neurites and cell body. Scale bar = 50 μm except days 9 and 21 in A, where bar = 75 μm. Figure reprinted from Streichert *et al.* (1997), by permission of Academic Press.

in vivo, where APR(4)s experience the prepupal peak yet survive through the pupal stage (Fig. 1). In hormone-free medium, APR(6)s survived as well as did APR(4)s (Fig. 3B), just as they do *in vivo* when the prepupal peak is prevented (Weeks *et al.*, 1992). However, addition of physiological concentrations of 20-HE to the culture medium caused a significant increase in the proportion of APR(6)s that underwent PCD; for example, in cultures exposed to 10 μg/ml 20-HE, 93% of APR(4)s survived for 20 days while only 25% of APR(6)s did so (Streichert *et al.*, 1997). As shown in Fig. 3C, the PCD of APRs culminates in profound fragmentation of the processes and cell body.

The experiments just described involved APRs that were removed from different larvae and cultured in different dishes. As an even more stringent test of the hypothesis that APRs respond autonomously to 20-HE based on intrinsic segmental identity, we prepared cocultures in which an APR(4) and APR(6) (labeled with DiA and DiI, respectively) from the *same larva* were placed in the *same culture dish* on day W0 and cultured in 10 μg/ml 20-HE. In every coculture tested, the APR(4) survived while the APR(6) underwent PCD (Streichert *et al.*, 1997). Thus, APRs removed from the same donor larva, differing only in segment of origin, exhibit strikingly different responses to an identical hormonal environment.

Three features of these experiments provided interesting additional insights into PCD in this system. First, although the cultures were prepared at low density, some APRs contacted other neurons. However, the presence or absence of contact had no influence on whether APR(4)s or APR(6)s exhibited the correct segment-specific responses to 20-HE. Second, the prepupal peak *in vivo* immediately follows day W0, whereas in culture the addition of 20-HE was delayed for 3 to 7 days to allow APRs to adhere and initiate growth. This delay in hormone exposure did not impair APRs' responses to 20-HE, just as dendritic regression and death of proleg motoneurons occurs normally *in vivo* when the prepupal 20-HE exposure is delayed by a number of days (Weeks and Truman, 1985). Thus, once motoneurons become pupally committed, this state of developmental readiness appears to persist indefinitely. Third, although APR(6)s *in vivo* normally experience both the prepupal elevation and subsequent decline in 20-HE before dying, a continuous elevation of 20-HE in culture is sufficient to trigger PCD. Although some developmental events during metamorphosis require that 20-HE levels decline after a rise (e.g., Woodard *et al.*, 1994), this is not the case for the death of APRs at pupation. This conclusion is also supported by *in vivo* experiments (Weeks and Truman, 1985).

The finding that APR(6)s die in response to an elevation of 20-HE at pupation is particularly interesting given that the APRs that die after adult emergence do so in response to a fall in 20-HE, as has now been shown both *in vivo* and *in vitro* (Zee and Weeks, 1998 and in preparation). Clearly, the adequate hormonal signal to activate PCD in APRs differs by segment and the developmental stage at which the APR dies. It has not been possible to dissociate the role of segmental identity *versus* developmental stage for this effect, but it is within the realm of possibility to do so by using hormonal manipulations to produce heterochronic mosaics; this technique permits neurons to be stage-advanced or stage-delayed relative to the rest of the nervous system and other tissues (e.g., Levine *et al.*, 1989; Jacobs and Weeks, 1990; Wilson and Kent, 1998). It may also be possible to block pharmacologically the PCD of APR(6)s at pupation (see below). Subsequently, it would be possible to determine whether APR(6)s spared from death at pupation undergo PCD in response to a subsequent elevation or decline in 20-HE. A finding that an elevation of 20-HE is the only adequate signal for PCD of APR(6)s would suggest that segmental identity determines the hormonal requirement, whereas a finding that a decline in 20-HE triggers PCD of APR(6)s spared at pupation would suggest that

developmental stage determines the adequate hormonal signal (with developmental stage being determined largely, if not exclusively, by the past history of hormonal exposure). Information of this type is crucial if we are to understand at the molecular level how intrinsic determinants of segmental identity and ecdysteroid actions coordinately regulate entry into the PCD pipeline.

4.2. Hormonal and Synthetic Requirements for PCD of APRs Cultured Late during the Prepupal Peak

The above experiments demonstrated unequivocally that 20-HE acts directly on APR(6)s to trigger PCD and that intrinsic segmental identity determines whether an APR lives or dies in response to the prepupal peak of ecdysteroids. These findings raised a number of interesting issues for further study, including the molecular basis of APRs' intrinsic segmental identity, and cellular and molecular events during the PCD of APRs. We have not yet addressed the nature of segmental identity, but have made progress in understanding the sequence of events during ecdysteroid-induced PCD of APR(6)s.

The experimental protocol used by Streichert *et al.* (1997)—culturing APR(6)s on day W0 and inducing PCD with 20-HE treatment—is not optimal for studying the specific sequence of events during PCD because the interval between 20-HE addition and the death of APR(6)s is somewhat long and variable: *in vivo*, five days separate the onset of the prepupal peak and the PCD of APR(6)s (Weeks *et al.*, 1992) whereas the mean time between 20-HE addition and the death of APR(6)s cultured at day W0 is approximately twelve days (Streichert *et al.*, 1997). This delay results in part from the use of fragmentation—a very late event during PCD (see below)—to score APRs as dead. Also, some cellular events appear to occur more slowly in culture, even though developing insects and cultured neurons are maintained at the same temperature. More recently, we have circumvented these issues by culturing APR(6)s later during development, when they have already undergone the required 20-HE exposure *in vivo* and are about to carry out the final execution phase of PCD.

In these experiments (Hoffman and Weeks, 1998a), DiI-labeled APR(4)s or APR(6)s were placed in culture at the time of pupal ecdysis (PE), the behavior used to shed the final larval cuticle on day P0 (Fig. 1). At PE, the prepupal peak has been completed and ecdysteroid levels are low. Previous *in vivo* studies found no segmental differences in the anatomy or electrophysiological properties of APRs at this developmental stage (Streichert and Weeks, 1995; Sandstrom and Weeks, 1998), even though some APRs will die within the next 24 to 48 hours. Consistent with these observations, APR(4)s and APR(6)s are indistinguishable when placed in culture at PE. In an example shown in Fig. 4A,B, the APR(4) and APR(6) both had large, ovoid somata with short neurites when placed in culture at PE in hormone-free medium. After 24 hours in culture, the APR(4) soma was still large and ovoid and had begun to extend processes (Fig. 4C), whereas the APR(6) soma was shrunken and rounded (Fig. 4D). Typically, after being placed in culture at PE, nearly all APR(4)s survive while a reliable population of APR(6)s (approximately 50%) undergo PCD within 24 hours, similar to the timing of events *in vivo* (Weeks *et al.*, 1992). The shrinkage and rounding of cultured APR(6) somata during PCD *in vitro* mimics the somatic morphology observed *in vivo* and is a robust, quantitative indicator of PCD (Hoffman and Weeks, 1998a). The shrunken, rounded phenotype is associated with the loss of staining with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a compound that is reduced to an insoluble purple product by mitochondrial succinate dehydrogenase and cytoplasmic NADH (Slater *et al.*, 1963; Berridge and Tan, 1993). Thus, loss of MTT staining indicates the loss of mito-

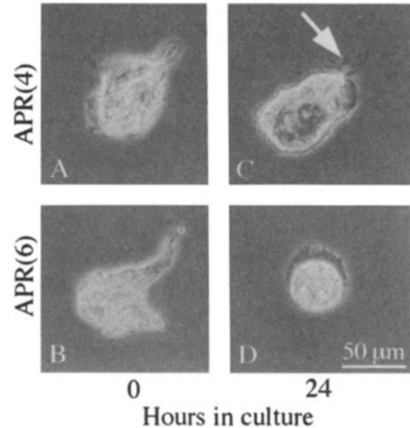


Figure 4. Segment-specific death of APRs cultured at pupal ecdysis. DiI-labeled APR(4)s and APR(6)s were placed in culture at pupal ecdysis (PE) in hormone-free medium and photographed at 0 and 24 h in culture. A. An APR(4) at 0 h in culture. B. An APR(6) at 0 h in culture. Both neurons had large, ovoid somata and a stump of neurite at this time. C. The same APR(4) shown in A, after 24 h in culture. The soma remained large and ovoid, and process outgrowth had begun (arrow). D. The same APR(6) shown in C, after 24 h in culture. The cell had become shrunken and rounded. Figure reprinted from Hoffman and Weeks (1998a), by permission of John Wiley and Sons, Inc.

chondrial function. Shrunken, rounded APR(6)s also exhibit the release of nuclear contents into the cytoplasm, as revealed by staining with the fluorescent dye, Hoechst 33342. Despite these dire cellular changes, staining with calcein AM and ethidium homodimer indicate that the plasma membrane of APR(6)s is still intact at 24 hours in culture, with fragmentation occurring considerably later (Hoffman and Weeks, 1998a). These observations underscore that PCD is a carefully orchestrated suicide that would be disrupted if membrane integrity were lost too soon.

APR(6)s that die after being placed in culture at PE offer an experimentally favorable system for studying the sequence of events during PCD; their death occurs relatively rapidly and during this period the neurons are highly accessible for pharmacological or hormonal treatments, optical imaging, electrophysiological recordings, or other techniques. In one series of experiments we investigated the requirements for 20-HE exposure and protein synthesis for the PCD of APR(6)s. We found that, although 20-HE is normally present at low levels at PE (Fig. 1), addition of 20-HE to the culture medium does not affect the proportion of APR(6)s that undergo PCD within 24 hours of PE. Furthermore, treatment with the protein synthesis inhibitor, cycloheximide (CHX), does not prevent the death of these APR(6)s. Thus, cultured APR(6)s that die within 24 hours of PE do not require further exposure to 20-HE or further protein synthesis to carry out PCD. This situation contrasts strikingly with APR(6)s that are cultured ~24 hours before PE, at stage "W3-noon"; at this time, both further exposure to 20-HE and further protein synthesis are required for the APR(6)s to undergo PCD (Hoffman and Weeks, 1998a). Similarly, CHX treatment during the prepupal peak blocks APR death *in vivo* (Weeks *et al.*, 1993). Thus, the final 24 hours of larval life represents an important developmental transition in the commitment of APR(6)s to undergo PCD.

Finally, in another series of experiments, we investigated the possible involvement of caspases (previously known as ICE-like proteases; Alnemri *et al.*, 1996) in the PCD of APR(6)s. The autocatalytic activation of caspases is a conserved aspect of PCD in species ranging from nematodes to humans (reviewed by Schwartz and Milligan, 1996; Porter *et al.*, 1997). Substrates of caspases include various cytoskeletal proteins and nuclear lamins. In other systems, such as PCD of sympathetic neurons after nerve growth factor withdrawal, treatment with caspase inhibitors can block death even after CHX treatment is no longer effective, suggesting that caspase activation is a very late step in PCD (reviewed by Deshmukh and Johnson, 1997). We found that treatment with Z-Asp-CH_2DCB, a general inhibitor of caspases (Dolle *et al.*, 1994; Nath *et al.*, 1996), significantly inhibited the ecdysteroid-induced shrinkage and loss of MTT staining in APR(6)s

cultured at W3-noon. In contrast, in APR(6)s cultured 24 hours later at PE, Z-Asp-CH_2DCB inhibited shrinkage but not the loss of MTT staining (Hoffman and Weeks, 1998b). Our data support a working model of 20-HE-induced PCD that includes two sequential phases of caspase activity in APR(6)s: an early phase that condemns mitochondria to a loss of function, and a later phase that mediates overt morphological changes such as cell body shrinkage and rounding (Hoffman and Weeks, 1998b and in preparation).

5. CONCLUSIONS

The concept of "metamorphosis as midlife crisis" highlights the spatially and temporally complex sequence of events that must be navigated successfully as an insect transforms from a larva to a pupa to an adult insect. The evolution of metamorphosis provided rich opportunities for biological diversity and success, but entailed new challenges when compared to the non-metamorphic lifestyle (reviewed by Sehnal *et al.*, 1996). Cells or structures with transient but critical functions contribute to embryonic development in many species (reviews by Burek and Oppenheim, 1996; Jacobson *et al.*, 1997) and the issue of what to do with leftover larval neurons during metamorphosis is the same issue on a grander scale. More globally, studies of phenotypic changes in neurons during metamorphosis, and the endocrine control of these events, provide an invaluable window into mechanisms of neural plasticity that span species and lifestyles. Hormonally-mediated changes in dendritic extent are hallmarks of steroid action in vertebrate animals (reviewed by García-Segura *et al.*, 1994; Kawata, 1995), and PCD is likewise critically important during development, adulthood and in disease states (reviewed by Thompson, 1995; Burek and Oppenheim, 1996). Studies of how the *Manduca* nervous system deals with leftover proleg neurons during metamorphosis provides a high-resolution view of a very circumscribed neurobiological problem. However, the detailed information available in this system is relevant to understanding developmental events in nervous systems that are numerically more complex and experimentally less accessible. Returning to the quote by Dave Barry at the beginning of this article, someday we may even understand the neural correlates of midlife crisis in humans, a time when adults look back nostalgically at their (larval) days of youth and fret about the levels of their own steroid hormones.

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9

DEVELOPMENT OF THE OLFACTORY SYSTEM IN THE AFRICAN CLAWED FROG, *XENOPUS LAEVIS*

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1. VERTEBRATE OLFACTORY EPITHELIUM AND OLFACTORY BULB

The olfactory system of adult mammals has been studied extensively (see Halász, 1990). The basic structure of the mammalian olfactory epithelium and olfactory bulb is outlined in this section to serve as background for the development and adult structure of the olfactory system in the African clawed frog, *Xenopus laevis*. The peripheral sensory tissue of the vertebrate olfactory system is the olfactory epithelium (see Farbman, 1992). The olfactory epithelium lines the dorsal roof, septum, and lateral turbinates of the caudal region of the mammalian nasal cavity. Another area of chemosensory tissue located at the floor of the nasal cavity of many mammals, snakes, and amphibians is the vomeronasal organ (see Farbman, 1992; Eisthen, 1997). In both the olfactory epithelium and vomeronasal organ, there are three basic cell types: olfactory receptor neurons, supporting cells, and basal cells. The receptor neurons are primary sensory neurons that are responsible for transducing odorant stimuli. They are bipolar neurons with an axon that projects to the olfactory bulb. The supporting cells can be secretory or ciliated cells that span the sensory epithelium from the apical border to the basal lamina. The basal cells are stem cells that have the ability to divide and differentiate into receptor cells during development and throughout the life of the organism.

The main olfactory bulb is the central target of the olfactory receptor neurons (see Shepherd and Greer, 1990; Farbman, 1992). In mammals, the bulb is a laminated structure consisting of olfactory nerve, glomerular, external plexiform, mitral cell, and granule cell layers. Axons of the olfactory receptor neurons synapse with output neurons, the mitral and tufted cells, and with interneurons, the periglomerular cells, and in some cases, these output and interneurons have reciprocal synaptic interactions. Another class of interneuron, the granule cell, also has reciprocal synapses with mitral cells. The electri-

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cal signal that results from this synaptic processing is carried out of the bulb to higher brain regions by the axons of mitral cells (Shepherd and Greer, 1990). The accessory olfactory system has a similar bulbar organization. The vomeronasal receptor neurons project to a separate region of the olfactory bulb, the accessory olfactory bulb, which has a similar organization and the same basic cell types found in the main olfactory bulb (see Farbman, 1992). Axons of the mitral cells in the accessory olfactory bulb also project to higher brain regions, but these regions generally differ from those of the main olfactory system (Scalia and Winans, 1975).

The goal of the current review is to outline the development of olfactory system by examining cellular factors that participate in the induction, differentiation, maturation, and remodeling of the peripheral and central olfactory system. This review will focus on studies in the African clawed frog, *Xenopus laevis*, which serves as an excellent model organism because cellular interactions can be studied through surgical manipulation in embryos. In addition, the basic mechanisms of neuronal remodeling can be examined at metamorphosis, in effect, a period of delayed embryogenesis (Fig. 1). Research on the cellular mechanisms provides a solid understanding of the remarkable plasticity involved in the embryonic and larval development of this system and establishes this system as an excellent model for future experiments focused on the molecular mechanisms that are responsible for the neural plasticity.

2. DEVELOPMENT AND ORIGIN OF OLFACTORY PLACODES

The olfactory epithelium in vertebrates is derived from bilateral thickenings of the cranial ectoderm, the olfactory placodes (Klein and Graziadei, 1983; see Farbman, 1992). The cells fated to be part of the olfactory placodes in *Xenopus* originate from a rim of tissue that resides along the anterior edge of the neural plate, the anterior neural ridge (Fig. 2) (Eagleson and Harris, 1990; Burd *et al.*, 1994). As the neural plate closes to form the neural tube, most of the anterior neural ridge remains on the exterior of the animal and forms an epithelial tissue called the sense plate at stage 20 (Fig. 2). This early sense plate is composed of two cell layers: a deeper layer and a superficial layer. The cells fated to form olfactory receptor neurons and basal cells are derived from the deeper cells in the sense plate, and those fated to form supporting cells are derived from the superficial cells of the sense plate (Klein and Graziadei, 1983; Burd *et al.*, 1994). The olfactory placodes begin to differentiate in two bilateral areas of the sense plate at stage 23 in *Xenopus* (Nieuwkoup and Faber, 1994). By stage 27/28, differentiated olfactory receptor neurons can be identified in the developing olfactory placodes (Fig. 3) (Klein and Graziadei, 1983), and at stage 30, the axons of the receptor neurons begin to contact the olfactory bulb (Byrd and Burd, 1991). Remarkably, the olfactory placodes can be removed as late as early larval stages (stage 40–41) and still regenerate (Stout and Graziadei, 1980; Byrd and Burd, 1993b) from adjacent sense plate cells (Burd *et al.*, 1994). Continued cell proliferation and differentiation within the olfactory placodes gradually form the olfactory epithelium in the principal cavity of the nasal capsule. By stage 37/38, the vomeronasal organ also begins to form (Fig. 3) (Nieuwkoop and Faber, 1994). Thus, *Xenopus* larvae have two regions of olfactory epithelium: principal cavity olfactory epithelium and vomeronasal epithelium. At metamorphosis, however, a new area of olfactory epithelium develops in *Xenopus* (see below).

There are no physiological studies that indicate when the *Xenopus* olfactory receptor neurons first respond to odorant stimuli. In the rat, however, Gesteland and colleagues

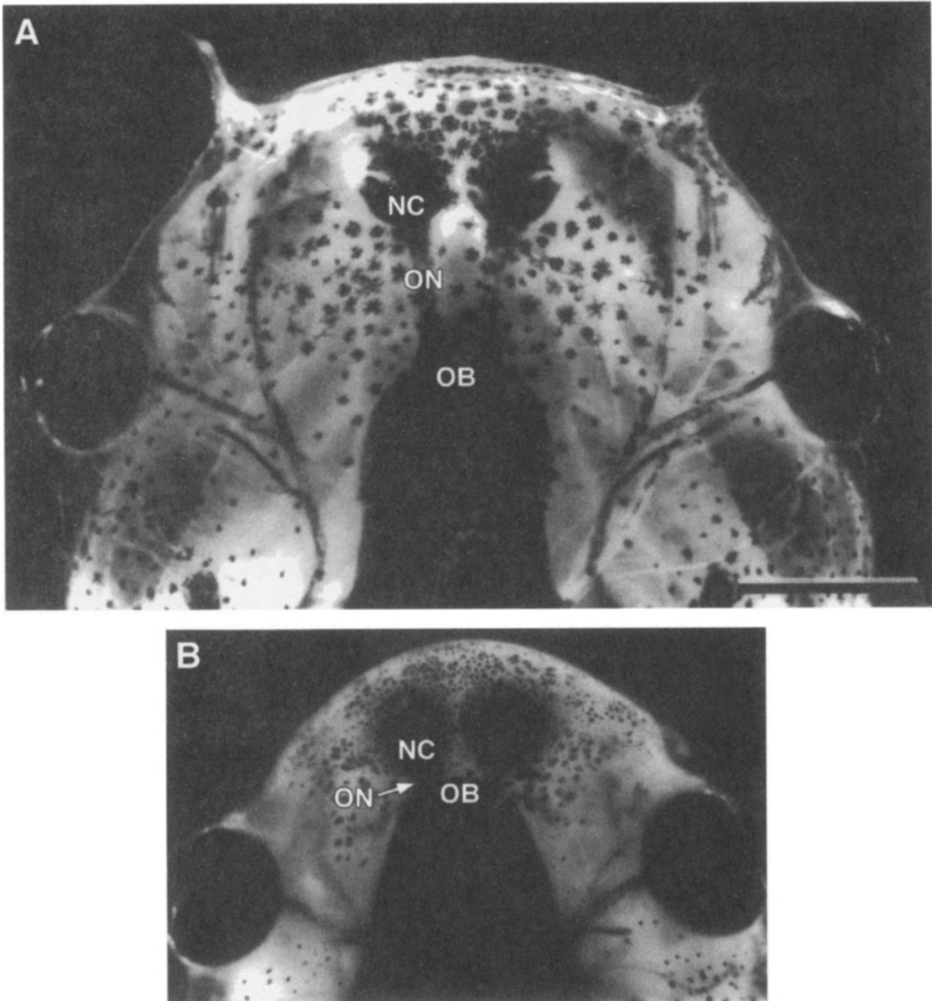

Figure 1. Heads of larval *Xenopus* at stage 58 (**A**) and stage 62 (**B**) illustrating the nasal capsule (NC), olfactory nerve (ON), and olfactory bulb (OB). Note that in the interval between stage 58 and stage 62 of metamorphic climax the head size and olfactory nerve length decrease. Figure modified from Burd (1991). Scale bar = 2 mm.

(1982) demonstrated that action potentials occur in olfactory receptor neurons at E16. The olfactory axons make synaptic contact with the olfactory bulb at E18, and only after this, at E21, are selective odorant responses recorded from olfactory receptor neurons. This temporal pattern suggests that feedback from the olfactory bulb may be needed to refine the responses of the receptor neurons.

3. DEVELOPMENT OF THE OLFACTORY BULB

The cells that give rise to the output neurons of the olfactory bulb, the mitral cells, are born beginning early in embryonic life at stage 11/12 in *Xenopus* (Fritz *et al.*, 1996). These neurons do not begin to differentiate until stage 32, however, just after innervation

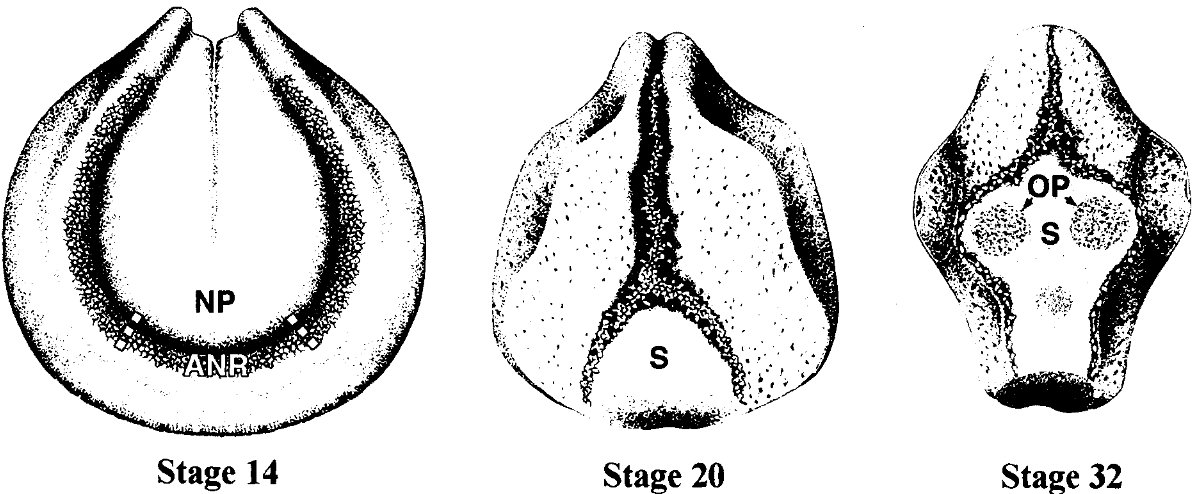

Figure 2. Schematic drawings of *Xenopus* embryos to illustrate the origin of the olfactory placodes. Embryos are drawn with the rostral edge facing out and the dorsal surface sitting up in the figure. Cells fated for the olfactory placodes originate in the anterior neural ridge (ANR) at the rostral edge of the neural plate (NP). As the neural tube closes, part of this tissue remains on the exterior of the animal as the sense plate (S). The olfactory placodes (OP) differentiate from and within the sense plate. This figure was modified from Fig. 10 in Drysdale and Elinson (1991).

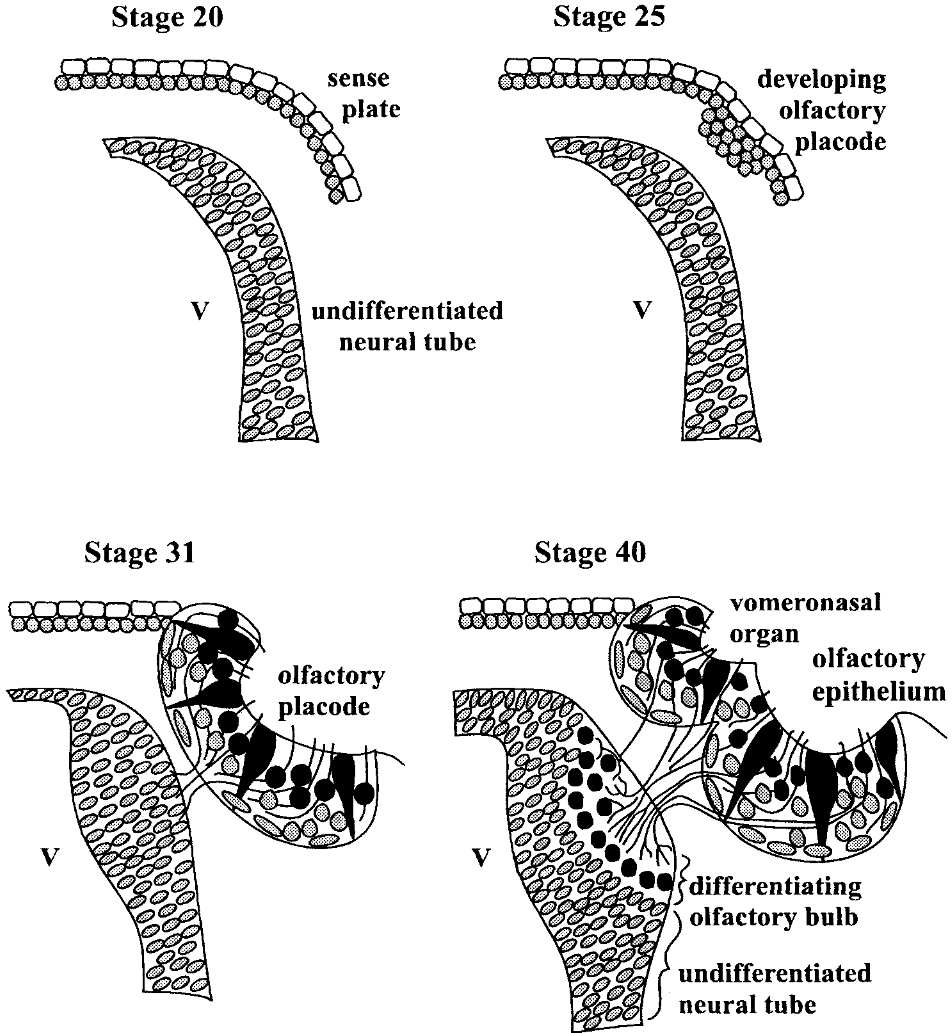

Figure 3. Schematic diagrams illustrating the development of the olfactory placode/olfactory epithelium and olfactory bulb in *Xenopus laevis* at embryonic stages 20, 25, and 31 and in a young larva at stage 40. The sense plate is formed by stage 20. It begins to thicken to form bilateral olfactory placodes, shown here at stage 25. By Stage 31, differentiated olfactory receptor cells (black spherical cells with processes) and differentiated supporting cells (large black cells) in the olfacory placode are present; the axons of the olfactory receptor cells contact the undifferentiated neural tube by stage 31. By stage 40, the olfactory epithelium of the principal cavity is separated from the vomeronasal organ, and the olfactory bulb has many differentiating mitral cells where olfactory axons have formed glomerular neuropil.

by the olfactory axons (Fig. 3) (Byrd and Burd, 1991). This is similar to development in rodents. The earliest olfactory axons penetrate deep into the undifferentiated telencephalon (rat: E13), contact the bulb precursor cells lining the ventricles, and stimulates a prolonged cell cycle in the precursor cells (Hinds, 1972; Gong and Shipley, 1995; de Carlos *et al.*, 1996). This cell-cell contact appears to stimulate differentiation of bulb neurons (Gong and Shipley, 1995), since Bayer (1983) finds the first postmitotic mitral cells occur at the same time, E13–14, in the rat. The olfactory axons ultimately retract from the deeper portions of the bulb to form the olfactory nerve layer.

In *Xenopus*, the olfactory nerve and glomerular layers form at stage 36, also the stage when the first synapses between olfactory axons and mitral cells are observed (Byrd and Burd, 1991). By early larval stages (stages 40–45), neurogenesis in the olfactory bulb includes the birth of mitral, periglomerular, and granule cells, and all layers of the olfactory bulb contain postmitotic neurons (Fritz *et al.*, 1996). At stage 44, all layers of the ventral olfactory bulb are present, and by stage 48/49, the laminar structure and synaptic organization is similar to that of adult frogs (Byrd and Burd, 1991). The structure that forms at these early larval stages will become the ventral olfactory bulb in post-metamorphic animals (Fritz *et al.*, 1996).

The adult structure of the olfactory bulb in *Xenopus* differs from that of the mammalian olfactory bulb in several respects (Fig. 4). First, olfactory projections from the periphery to the olfactory bulb are not strictly ipsilateral in *Xenopus* as they are in mammals. A change in the bulb organization occurs at stage 50, with the right and left olfactory bulbs joining at the midline to produce a fused olfactory bulb (Fig. 4). By the onset of metamorphic climax (stage 58), many olfactory axons from one side of the animal cross within the olfactory nerve layer at the midline and distribute throughout the nerve and glomerular layer on the contralateral side (Byrd and Burd, 1993a). Second, there are afferent fibers from some receptor neurons, neurons of the terminal nerve ganglion, and non-receptor neurons called globular cells that project directly through olfactory bulb to brain targets in the diencephalon and mesencephalon, and early in larval life, also project to the metencephalon and myelencephalon (Hofmann and Meyer, 1991; Venus, Wolff, and Burd, unpublished observations). These "extra-bulbar" projections do not exist in mammals. A third difference between the bulb of *Xenopus* and mammals is the loose glomerular organization in *Xenopus*. While the glomeruli are clearly outlined by periglomerular, tufted, and glial cells in mammals, there are few of these cell types in *Xenopus* and the glomerular neuropil is not clearly defined (compare Burd, 1993 with Byrd and Burd, 1991). Fourth, mitral cells have a single, unbranched primary dendrite that enters one glomerulus in mammals, but in *Xenopus laevis* (Burd, unpublished observations) and in *Rana pipiens* (Scalia *et al.*, 1991), the primary dendrites often branch widely to enter two different regions of the glomerular layer. If olfactory receptor neurons with similar physiological responses and/or the same odorant receptor gene project to restricted glomeruli, as in mammals (Imamura *et al.*, 1992; Mombaerts *et al.*, 1996, Wang *et al.*, 1998), this could mean that mitral cells in frogs receive multiple types of odorant stimuli. Too little information on the physiological responses in *Xenopus* exist to form firm conclusions on this issue. A fifth difference between the bulb of mammals and that of *Xenopus* is the distribution of mitral cells and the organization of the external plexiform layer. In mammals, the mitral cell layer and the external plexiform layer are distinct, while in *Xenopus*, these two layers are mixed together with mitral cells scattered throughout the external plexiform layer (Byrd and Burd, 1991). Lastly, the distribution of the candidate neurotransmitters differs between *Xenopus* and mammals, as demonstrated with immunocytochemistry for the neurotransmitter or its synthetic enzymes. For example, while tyrosine hydroxylase immunoreactivity is present in the *Xenopus* bulb (Burd *et al.*, 1987), as it is in the rat bulb (Baker *et al.*, 1983), the immunopositive neurons are present in the glomerular and external plexiform/mitral cell layer in *Xenopus*, while they are restricted to the glomerular layer in rodents. Furthermore, serotonin fibers have a major distribution around glomeruli in mammals (McLean and Shipley, 1987), but do not project to the glomerular layer in *Xenopus* (Burd *et al.*, 1987). In addition, immunoreactivity for substance P and enkephalin have been located within bulb neurons in hamster and other species of mammals (Davis *et al.*, 1982; Burd *et al.*, 1982; Shepherd and Greer,

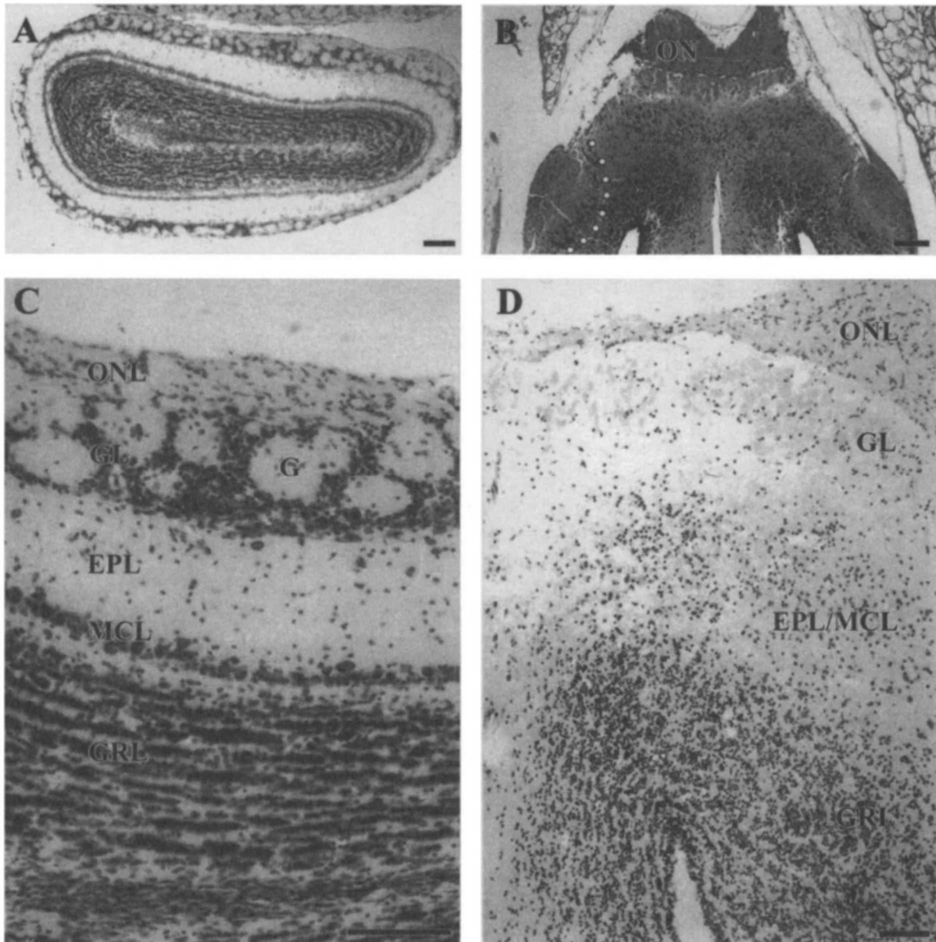

Figure 4. Comparison of the structure of the mouse and *Xenopus* olfactory bulbs. **A.** Coronal section of the olfactory bulb of an adult mouse stained with cresyl violet; dorsal is to the left and medial is at the top of the figure. **B.** Horizontal section of the olfactory bulb from a stage 58 *Xenopus* larva stained with hemotoxilyn and eosin; anterior is at the top of the figure. Note that the left and right sides of the *Xenopus* bulb are fused; in mice, the right and left bulbs are totally separated. Also, note that the bulb layers in *Xenopus* are organized as lamina from rostral to caudal, while in the mouse (A) the layers form concentric lamina around the bulb. The accessory olfactory bulbs are bilateral structures; the left accessory olfactory bulb is outlined with white dots. **C.** Higher magnification of the olfactory bulb of an adult mouse stained with cresyl violet; the medial edge of the bulb faces up. Note the layers of the bulb: olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), and granule cell layer (GRL). In the mouse, the glomeruli (example at G) are clearly defined by interneurons and glial cells. **D.** Horizontal section from the olfactory bulb of an adult *Xenopus* stained with cresyl violet. Note that the MCL is blended with the EPL in *Xenopus*. Also, note that glomeruli are not defined by neurons or glial cells in *Xenopus*. Scale bars: **A** = 20 µm; **B**, **C**, **D** = 10 µm.

1990), but immunoreactivity for these peptides is not present in the *Xenopus* bulb, even though stained neurons are present in other brain areas (Burd, unpublished observations). Collectively, these differences in the structure of the *Xenopus* bulb suggest that odorant information is likely to receive less-specific synaptic processing within the bulb than has been reported for mammals (Shepherd and Greer, 1990).

4. ROLE OF SENSORY AFFERENTS IN OLFACTORY BULB DEVELOPMENT

In most sensory systems, the sensory afferents play a major role in the development of their target (Kollros, 1953; Selleck and Steller, 1991; Parks, 1979; Born and Rubel, 1985; Hildebrand *et al.*, 1979; Oland and Tolbert, 1987; Tolbert and Sirianni, 1990). In the amphibian olfactory system, cellular interactions between olfactory axons and the olfactory bulb are critical for the formation of the bulb; without innervation from the olfactory axons, the olfactory bulb does not form (Piatt, 1951; Clairambault, 1976; Graziadei and Monti Graziadei, 1992; Byrd and Burd, 1993b). In mammals, "experiments-of-nature" have provided insight into a similar need for olfactory axon innervation for normal bulb development. For example, humans with a null mutation in the Kallmann gene fail to make an essential cell adhesion molecule that is normally expressed in the olfactory bulb (Franco *et al.*, 1991). As a result, the olfactory axons fail to innervate the telencephalic vesicle, and the bulbs in these individuals do not form. Also, the olfactory axons fail to contact the rostral neural tube in mice with a null mutation in the *Gli3* gene (*Pdn/Pdn* mice), and the bulb does not form (Naruse *et al.*, 1994). There may be some initial differentiation of bulb neurons in the absence of olfactory axons, however, since Mori *et al.* (1997) demonstrated the presence of a few neurons with the bulb-specific, neuronal marker, O-CAM, in these *Pdn/Pdn* mice.

In *Xenopus* during normal development, the number of olfactory axons (Burd, 1991) and the number of mitral/tufted cells increase steadily. In fact, the number of olfactory axons is correlated with the number of mitral/tufted cells throughout larval periods, with five olfactory axons to every mitral/tufted cell (Byrd and Burd, 1991). Furthermore, if one olfactory placode is removed during embryonic stages, there is a corresponding decrease in the number of mitral/tufted cells (Byrd and Burd, 1993b). In the moth *Manduca sexta*, the target of olfactory receptor neurons, the antennal lobe, develops partially in the absence of olfactory axons, but normal glomeruli do not form. Furthermore, partial deafferentation results in the formation of a graded response; once above threshold, more afferents yield more glomeruli (Tolbert and Sirianni, 1990). The quantitative relationship in *Xenopus* and the graded response in *Manduca* suggest that the olfactory axons provide an essential growth factor or stimulus through cell-cell contact that is required for normal bulb development and regulated by axon number. Transplantation of a supernumerary olfactory placode, to increase the number of olfactory axons that innervate the bulb in *Xenopus*, however, does not produce a larger olfactory bulb (Byrd and Burd, 1993a). In fact, the transplant does not contribute extra afferent fibers, and it seems that the number of normal olfactory axons is reduced by the transplant. This finding suggests that the bulb is able to regulate the number of innervating afferent fibers. In adult rodents, the olfactory bulb has been shown to be essential for maintenance of mature olfactory receptor neurons; olfactory receptor neurons are able to mature, but have reduced life spans following removal of their target, the olfactory bulb (Schwob *et al.*, 1992).

Transection of the olfactory nerve in larval *Xenopus* (Burd and Sein, 1998) and naris closure in early postnatal rats (Frazier and Brunjes, 1988; Frazier-Cierpial and Brunjes, 1989) and opossum (Cummings *et al.*, 1997) result in a reduced size of the olfactory bulb. In the rat, naris closure results in a reduction in the number of periglomerular, external tufted, and granule cells, but not mitral cells. In the opossum, which is born at a more premature state, mitral cells are also affected by naris closure. These neuronal populations could be reduced through an effect on cell genesis of neurons still being generated or on neuronal maturation and survival. In rats with naris closure and in larval *Xenopus* with transection of the olfactory nerve, the number of precursor cells that divide along the ventricular zone is unaffected, but in both cases, the number of newly generated neurons that mature is reduced (Frazier-Cierpial and Brunjes, 1989; Burd and Sein, 1998). A required trophic factor from the olfactory axons may be missing following nerve transection and naris closure. In addition, neuronal activity is reduced in the bulb neurons following naris closure (Guthrie *et al.*, 1990; Philpot *et al.*, 1997); thus, the activity in bulb neurons may play a role in their survival.

5. ROLE OF THYROID HORMONE IN NEURAL PLASTICITY AT METAMORPHOSIS

In many neural systems, hormones have been shown to play an important role in the formation and differentiation of neurons (Grave, 1977; Arnold and Gorski, 1984; Weeks and Levine, 1990). Thyroid hormone, in particular, has been shown to play a crucial role in development of the vertebrate nervous system by inducing a variety of anatomical, biochemical, and physiological events during critical periods of neural development (Grave, 1977; Lauder and Krebs, 1986; Dussault and Ruel, 1987). For example, human newborns with hypothyroidism will become mentally retarded unless treatment with thyroid hormone begins soon after birth (Green, 1987). Studies on rodents with pharmacologically-induced hypothyroidism have shown that thyroid hormone is an important factor for the developing olfactory system (Segovia *et al.*, 1982; Paternostro and Meisami, 1991, 1994, 1996), and plays a major role in attainment of the normal structure and function of the adult olfactory system in mice (Mackay-Sim and Beard, 1987; Beard and Mackay-Sim, 1987) and humans (McConnell *et al.*, 1975).

In addition, thyroid hormone is essential for metamorphosis in a number of amphibians and fish, and plays a role in neuronal remodeling and behavioral changes that occur at metamorphosis (Dodd and Dodd, 1976; Kollros, 1981; White and Nicoll, 1981; Hasler and Scholz, 1983). In *Xenopus*, the titers of thyroid hormone change during larval life, rising at the end of premetamorphosis (stage 54), increasing dramatically after the onset of metamorphic climax (stage 58), peaking during metamorphic climax (stage 62), and falling to low levels after metamorphosis where it remains throughout adulthood (Fig. 5) (Leloup and Buscaglia, 1977; Regard *et al.*, 1978; White and Nicoll, 1981). The term "thyroid hormone" actually is used to refer to two forms of the hormone: thyroxine (T_4) and triiodothyronine (T_3). T_4 is the major hormone secreted from the thyroid gland; it circulates to peripheral tissues where it is converted to the more active form of the hormone, T_3 (see Green, 1987), and binds to thyroid hormone receptors.

Xenopus contains two classes of thyroid hormone receptors (TRα and TRβ) that are active during metamorphosis (Yaoita and Brown, 1990; Yaoita *et al.*, 1990). Some of the TRα protein is present maternally, but the level of TRα protein increases during metamorphosis and remains constant until climax (stage 62). TRβ protein, on the other hand,

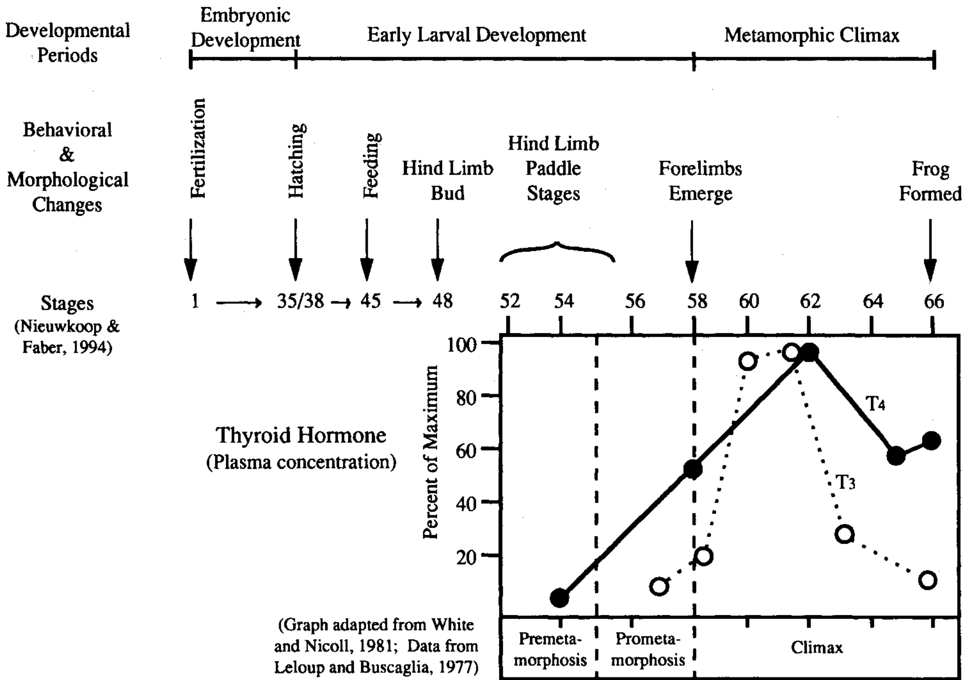

Figure 5. Developmental time-line for *Xenopus laevis* that includes information about the major developmental periods, behavioral and morphological changes, developmental stages, and plasma level of thyroid hormone (T4 and T3). The stages were defined by Nieuwkoop and Faber (see 1994) and the plasma levels were determined by Leloup and Buscaglia (1977). The graph of plasma levels of thyroid hormone was modified from figure 4 in White and Nicoll (1981).

is detectable only at the onset of metamorphosis (stage 52) and its level increases progressively to reach a plateau at climax (Eliceiri and Brown, 1994). Activation of these receptors by thyroid hormone leads to the regulation of many genes that are responsible for producing the cellular and biochemical changes observed at metamorphosis (Kanamori and Brown, 1992; Shi, 1996).

Among the tissues that undergo significant change at metamorphosis in response to thyroid hormone is the peripheral olfactory system (Burd, 1990). The expression of high levels of thyroid hormone receptors in the nasal capsule in *Xenopus* indicates that thyroid hormone is likely to act directly on the olfactory epithelium at metamorphosis (Kawahara *et al.*, 1991). Also, in the developing olfactory epithelium of *Xenopus*, hypothyroidism results in significantly fewer mature olfactory receptor neurons, while precocious thyroid hormone stimulates an increase in cell division as well as neuronal maturation (Burd,1990, 1992; Burd and Chvatal, 1993). Changes in olfactory behaviors, though expected, have not been reported in *Xenopus* at metamorphosis, but have been studied extensively in salmon undergoing metamorphosis. For example, early exposure to thyroid hormone stimulates imprinting on stream odors in young, migratory salmon, and this odor memory (and preference) is retained for 10 months without further odor stimuli or hormonal treatment (Hasler and Scholz, 1983). In addition, results from Nevitt *et al.* (1994) using patch clamp recordings on odor-imprinted smolt salmon indicate that some part of the odor memory may reside within the olfactory receptor cells. Thus,

thyroid hormone is responsible for stimulating significant changes in the function of the salmon olfactory system at metamorphosis.

6. PLASTICITY IN THE OLFACTORY SYSTEM DURING METAMORPHOSIS

The olfactory epithelium of the African clawed frog, *Xenopus laevis,* demonstrates remarkable neural plasticity during metamorphosis (see Reiss and Burd, 1997b). The nasal capsule expands from two areas of larval olfactory epithelium, housed in the principal cavity and the vomeronasal cavity, to three areas of adult olfactory epithelium, located in the principal cavity, vomeronasal cavity and the newly formed middle cavity (Fig. 6) (Föske, 1934; Paterson, 1939). The function of the principal cavity changes at metamorphosis in *Xenopus*. In the larva, the principal cavity is exposed to water and presumably is able to sense water-borne odorants. After metamorphosis, the structure of the principal cavity changes to exclude water from entering most of the cavity and to permit air to enter the cavity (Altner, 1962; Venus and Burd, 1998). This is the likely pattern followed by most frogs at metamorphosis as they develop the ability to leave water for terrestrial environments. *Xenopus*, on the other hand, retains its aquatic lifestyle after metamorphosis. Interestingly, *Xenopus* also develops a new area of olfactory epithelium in the middle cavity that appears to assume the role of sensing water-borne odorants. Young postmetamorphic and adult frogs exposed to dye-stained water for several days show dark staining of the olfactory epithelium in the vomeronasal and

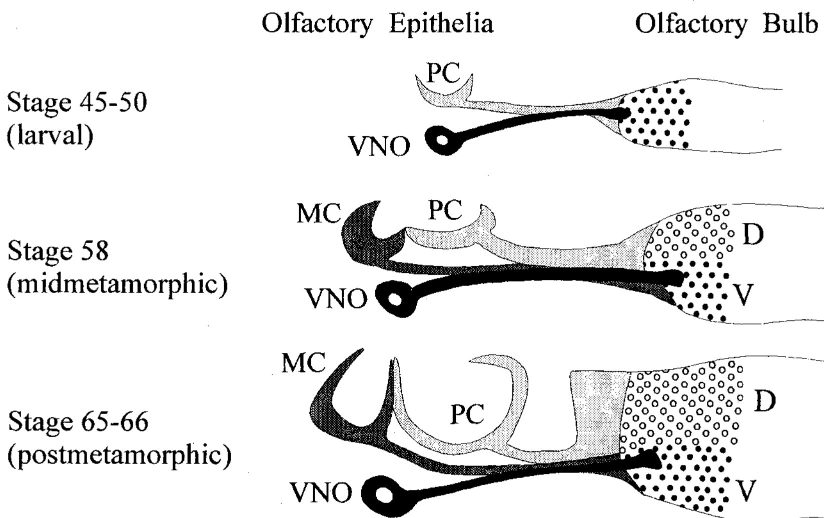

Figure 6. Schematic diagram of the changes that occur in the olfactory system of *Xenopus laevis* during larval development. In the postmetamorphic frog, there are three regions of olfactory epithelium located in the principal cavity (PC), middle cavity (MC), and vomeronasal cavity (VNO), and two regions of the main olfactory bulb, dorsal (D) and ventral (V). Note that the MC forms in the midmetamorphic stages. Also note that neurons of the dorsal bulb (clear circles) are not born until midmetamorphic stages and the PC axons leave their larval target (filled circles) to innervate the dorsal bulb (open circles) from midmetamorphic stages to adulthood. The MC axons innervate the old larval bulb, which now becomes the ventral olfactory bulb. The VNO and its axons do not undergo significant changes at metamorphosis. Figure modified from Key (1986).

middle cavitites, but very little staining is observed in the principal cavity (Venus and Burd, 1998).

As these changes in water exposure occur at metamorphosis in the larval principal cavity, there are also significant transformations in anatomy, biochemistry, and presumably physiology. Anatomically, the cell types in this cavity differ in type and fine structure before and after metamorphosis. The larval principal cavity contains ciliated and microvillar receptor cells and ciliated and secretory supporting cells. The adult principal cavity, in contrast, contains only ciliated receptor cells and secretory supporting cells (Hansen *et al.*, 1998).

Additional anatomical differences are observed in the projections of receptor neurons from the larval and adult principal cavities. Olfactory axons from the larval principal cavity project to a small olfactory bulb before metamorphosis, but at metamorphosis the axonal projections from the receptor neurons in the principal cavity are lost from this larval target. The principal cavity axons project to a new region of the olfactory bulb in the frog (Weiss, 1986; Reiss and Burd, 1997 a,b). In fact, this region of the olfactory bulb, the dorsal olfactory bulb of the adult, forms *de novo* at metamorphosis (Fritz *et al.*, 1996). At stage 54, for the first time, neurons in the dorsal olfactory bulb are generated. The generation of neurons in the dorsal bulb, however, does not follow the normal developmental pattern in which large output neurons in a given brain area are born before smaller interneurons. Instead, both output neurons and interneurons in the dorsal olfactory bulb are born at the same time. Neurons in the ventral bulb generally follow the more typical pattern of neurogenesis (Fritz *et al.*, 1996). In both the dorsal and ventral bulb, neurogenesis continues at least through the end of metamorphosis (Fritz *et al.* 1996).

Biochemically, metamorphosis of the principal cavity is accompanied by changes in cell surface and cytoplasmic molecules. For example, lectin binding characteristics change in the axons of the principal cavity from heavy soybean agglutinin (SBA) staining in larva to very light staining in adults (Key, 1986; Hofman and Meyer, 1991; Reiss and Burd, 1997a). In addition, the monoclonal antibody, E7, intensively stains the cell body and dendrite of olfactory receptor neurons in the larval principal cavity, but staining is not observed in the cell body and dendrite of receptor neurons of the adult principal cavity (Petti *et al.*, 1999). In fact, the loss of the E7 staining pattern begins in the rostro-medial area of the principal cavity (oral cupula of the principal cavity) and gradually expands to include all area of the principal cavity. The transition in the E7 staining may be correlated with a similar transition in the ultrastructural changes, but this remains to be determined.

While the principal cavity is undergoing major structural and biochemical changes, the middle cavity, which forms *de novo* at metamorphosis, assumes the fine structure, histochemical characteristics, axonal projections, and presumed functional characteristics of the larval, water-sensing principal cavity (Reiss and Burd, 1997a). As in the larval principal cavity, the adult middle cavity olfactory epithelium has ciliated and microvillar receptor neurons and ciliated and secretory supporting cells (Hansen *et al.*, 1998). In this respect, the receptor types in the middle cavity are like that in fish (Eisthen, 1997), which sense odors—especially amino acids (Hara, 1994)—in water. The adult middle cavity has been shown to respond to amino acid chemical stimuli (Schild *et al.*, 1990; Kruzhalov, 1995) and is exposed to water stimuli (Altner, 1962; Venus and Burd, 1998). In addition, as in the larval principal cavity, the axons of the olfactory receptor neurons in the middle cavity have intense staining with the lectin SBA (Key and Giordi, 1986; Hofmann and

Meyer, 1991; Reiss and Burd, 1997a) and the receptor neuron cell bodies and dendrites stain brightly with the E7 monoclonal antibody (Petti *et al.*, 1999). Finally, the axons of olfactory receptor neurons of the middle cavity project to the same target in the olfactory bulb that has been vacated by the larval, water-sensing receptor neurons of the principal cavity (Weiss, 1986; Reiss and Burd, 1997a). Collectively, the above observations suggest that water-sensing olfactory epithelium in *Xenopus* requires specific cell types and biochemical features, and that to sense odorants in air it is necessary to produce major transformations in the cells and chemical nature of the olfactory epithelium. In addition, an interesting finding is that the ventral olfactory bulb appears to be specialized for processing water-borne stimuli, because it receives projections of axons from the larval principal cavity and the adult middle cavity. This finding should make it possible to dissect the projection patterns of olfactory axons sensitive to particular classes of odorants.

In 1991, Buck and Axel discovered a large multigene family in rats that is expressed in the olfactory epithelium, but not other tissues, and appears to encode putative odorant receptors. The genes of this family were predicted to encode a seven transmembrane domain protein with a G-protein activation site. Firestein and his colleagues (Zhao *et al.*, 1998) provided the first convincing evidence for functional expression of odorant receptors in rodents and proved that these receptors have ability to participate in odorant transduction. *Xenopus* has two major subclasses of odorant receptors that resemble mammalian and fish odorant receptor genes (Freitag *et al.*, 1995). In adult *Xenopus*, the expression of the mammalian-like receptor genes occurs in the air-sensing cavity (principal cavity), while the fish-like receptor genes are expressed in the water-sensing cavity (middle cavity) (Freitag *et al.*, 1995). Preliminary results from Hansen *et al.* (1996) and Metzler *et al.* (1998) indicate that both forms of the receptor might be expressed in the principal cavity of the larva. This would suggest that expression of the fish-like odorant receptor genes is lost from the principal cavity at metamorphosis, coincident with the loss of the microvillar receptor cells from the principal cavity. The middle cavity may develop as the larval principal cavity with initial expression of the mammalian-like receptor gene, but this gene subclass would have to be lost during the late stages of larval development.

7. PLASTICITY IN AXON TARGETING TO THE OLFACTORY BULB AT METAMORPHOSIS

The identification of factors that control axon targeting is a major interest in developmental neurobiology, with investigators trying to determine whether axons are specifically targeted to a given brain area or whether competition plays a role in directing axons to appropriate targets. This is also an exciting area to investigators working on the olfactory system. For example, Mombaerts and coworkers (1996) showed that axons of olfactory receptor neurons that express a particular type of odorant receptor gene are distributed broadly within one of the four zones in the olfactory epithelium in mice, but the axons of all receptor neurons with the same odorant receptor project to the same few glomeruli in the olfactory bulb. This projection pattern is consistent across different individual mice. In addition, the odorant receptor genes are expressed before olfactory axons contact the olfactory bulb, and axons appear to target to their respective glomeruli with little or no aberrant projections during development. Using transgenic mice with altered

odorant receptor gene expression, Mombaerts *et al.* (1996) and Wang *et al.* (1998) suggest that the odorant receptors may participate, in some way, in target selection by olfactory axons.

In *Xenopus*, the presumed odorant selectivity of the principal cavity epithelium changes when the cavity goes from water exposure to air exposure at metamorphosis. Also at this time, the projection patterns of olfactory axons from the principal cavity change from innervation of the tissue that will become the ventral bulb to innervation of the newly formed dorsal bulb. At the time when the principal cavity axons are lost from the ventral bulb, middle cavity olfactory axons are innervating this bulb region. Thus, the principal cavity axons could be forced from their ventral target by competition from the middle cavity axons. To test this hypothesis, the middle cavity epithelium and its axons were removed in young larvae. This lesion had little effect on the targeting of the principal cavity axons, even though the lesion eliminates innervation of the ventral bulb and makes available a large area of potential target (Reiss and Burd, 1997a); the adult principal-cavity axons still project directly to the dorsal olfactory bulb (Reiss and Burd, 1997b). Thus, receptor neurons, which presumably sense air-borne odorants and are likely to express mammalian-like odorant receptor genes, are specifically directed to one region of the olfactory bulb. This implies that, at metamorphosis, there must be a change in some feature of principal-cavity axons or their target neurons that either attract the principal-cavity axons to the dorsal bulb or repel these axons from the ventral bulb.

8. SUMMARY

In summary, as outlined in this chapter, the olfactory system in *Xenopus laevis* has many similarities with as well as differences from the structure and function of the fish and mammalian olfactory systems. The neural plasticity that occurs in the olfactory epithelium and olfactory bulb at metamorphosis in *Xenopus* (Table 1) serves as an excellent model for studies on the structural, molecular, biochemical, physiological, and behavioral requirements for olfactory function in aquatic and terrestrial environments. In addition, the accessability of various components of the olfactory system during embryonic and larval development make *Xenopus* particularly attractive for experiments that require experimental manipulation. Future experiments will continue to address the mechanisms involved in the development of this system at these two stages of life.

Table 1. Major Changes that Occur in the Olfactory Epithelium in *Xenopus laevis* at Metamorphosis

	Larval PC	Adult MC	Adult PC
function	water sensing	water sensing	air sensing
receptor cell ultrastructure	ciliated and microvillar	ciliated and microvillar	ciliated
biochemistry	SBA+ E7+	SBA+ E7+	SBA– E7–
olfactory axon projections	small OB (becomes ventral OB)	ventral OB	dorsal OB
odorant receptors	both types (?)	fish-like	mammalian-like

PC = principal cavity, MC = middle cavity, SBA = soy bean agglutinin, E7 = monoclonal antibody E7, OB = olfactory bulb. For references, see the text.

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10

AFFERENT REGULATION OF DEVELOPMENTAL FATE IN THE SONGBIRD TELENCEPHALON

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1. INTRODUCTION

Songbirds are among few vertebrates other than humans that learn the sounds used by their species to communicate during a sensitive period of postnatal development. Unlike humans, however, where both sexes learn and use vocal communication, in many species of songbird only males learn to produce song. Zebra finches provide a particularly striking example of a sex difference in songbird vocal behavior. Males learn a vocal pattern during juvenile and early adult development, while females do not.

2. SONG LEARNING IS DEVELOPMENTALLY REGULATED

Zebra finches reach sexual maturity (adulthood) by 90 days post-hatch. Song learning, which occurs largely between 20 and 70 days post-hatch, is a developmentally regulated form of learning (Immelmann, 1969). Zebra finches spend the first three weeks of post-hatch life as nestlings, being fed by their parents while remaining in the nest. As they enter the fourth week of life (around 20 days of age), zebra finches will attempt to leave the nest and fly for the first time. Song learning appears to begin during this fledgling stage of development (Arnold, 1975; Bohner, 1990). As fledgling males begin to interact socially with other birds, they are exposed to the song pattern of an adult (typically the father) that is committed to memory, a process termed "auditory learning". An adult song pattern consists of a series of 5–10 different note types that are repeated in a stereotyped order each time the song is produced (see example in Fig. 1).

By 30–35 days of age, juvenile males begin a second phase of vocal learning, termed "sensory-motor learning." During this phase males begin to vocalize. Initially, their vocalizations are highly variable, lacking consistency in note types and note sequence (Arnold, 1975). Over time, and requiring auditory feedback of their own utterances (Price, 1979), young males refine their vocal patterns such that the patterns eventually resemble the

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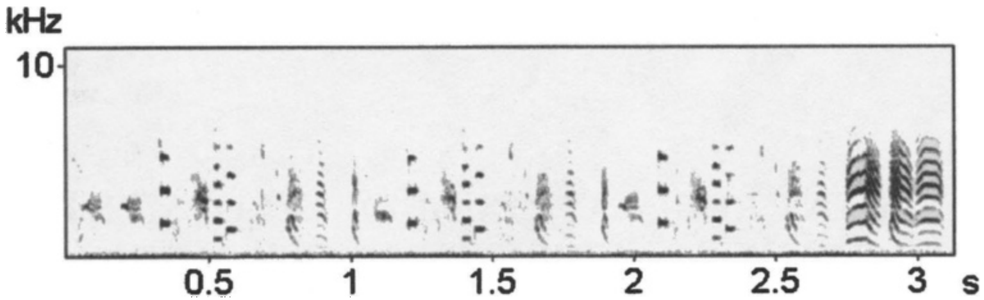

Figure 1. Sonogram (a graph of frequency over time) of an adult male zebra finch song bout. Shown is a bout of singing that contained three repetitions of the bird's vocal pattern followed by three call notes.

paternal song heard earlier in life. By 90 days of age, males are producing a specific pattern of notes in a stereotyped fashion that is unaltered for the remainder of life.

Early exposure to a song model and auditory feedback are crucial to zebra finch song behavior. If young males are deprived of a song tutor or deafened during development they will produce highly abnormal songs (Immelman, 1969; Price, 1979). Evidence of closure of the sensitive period for learning is best observed in tutor-deprived birds—if males are deprived of a song model during development but given the opportunity to hear normal song as adults, they are unable to modify their abnormal vocalizations (Immelman, 1969). However, even though zebra finches are unable to learn song as adults, they still require auditory feedback for the maintenance of normal adult song (Nordeen and Nordeen, 1992).

In contrast to learned song by males, female zebra finches do not learn to produce a vocal pattern, and their calls also show no evidence of a learned component (Simpson and Vicario, 1990). This behavioral dimorphism finds dramatic expression in the zebra finch brain. Male brains contain an anatomically prominent network of brain regions (nuclei) that control vocal behavior (Nottebohm *et al.*, 1976, 1982; Bottjer *et al.*, 1989; see Fig. 2). In non-singing females, vocal control brain regions and their axonal pathways are either absent or diminished in size and neuron number (Bottjer *et al.*, 1985; Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989). It is important to note, however, that while females do not learn to produce vocal behavior themselves, song-control circuitry in the female brain does serve a purpose. That is, the female song system may be involved in the perceptual recognition of song patterns and it could be one mechanism used by females to recognize potential mates (Brenowitz, 1991).

2.1. Sexually Dimorphic Fates for Song-Control Brain Regions during Development

In males, song control brain regions undergo substantial changes in size, neuron number, and axonal connectivity *during* song learning, and it has been suggested that these developmental changes may be timed so as to coincide with the period of vocal learning (Bottjer *et al.*, 1985; Herrmann and Bischof, 1986). Interestingly, the emergence of sexual dimorphism in the song system reinforces the idea that morphological development is timed so as to regulate the ability of juvenile birds to learn song. For example, some female song regions are monomorphic to those of males at the beginning of song learning (most notably lMAN and RA), but these regions then atrophy as the juvenile

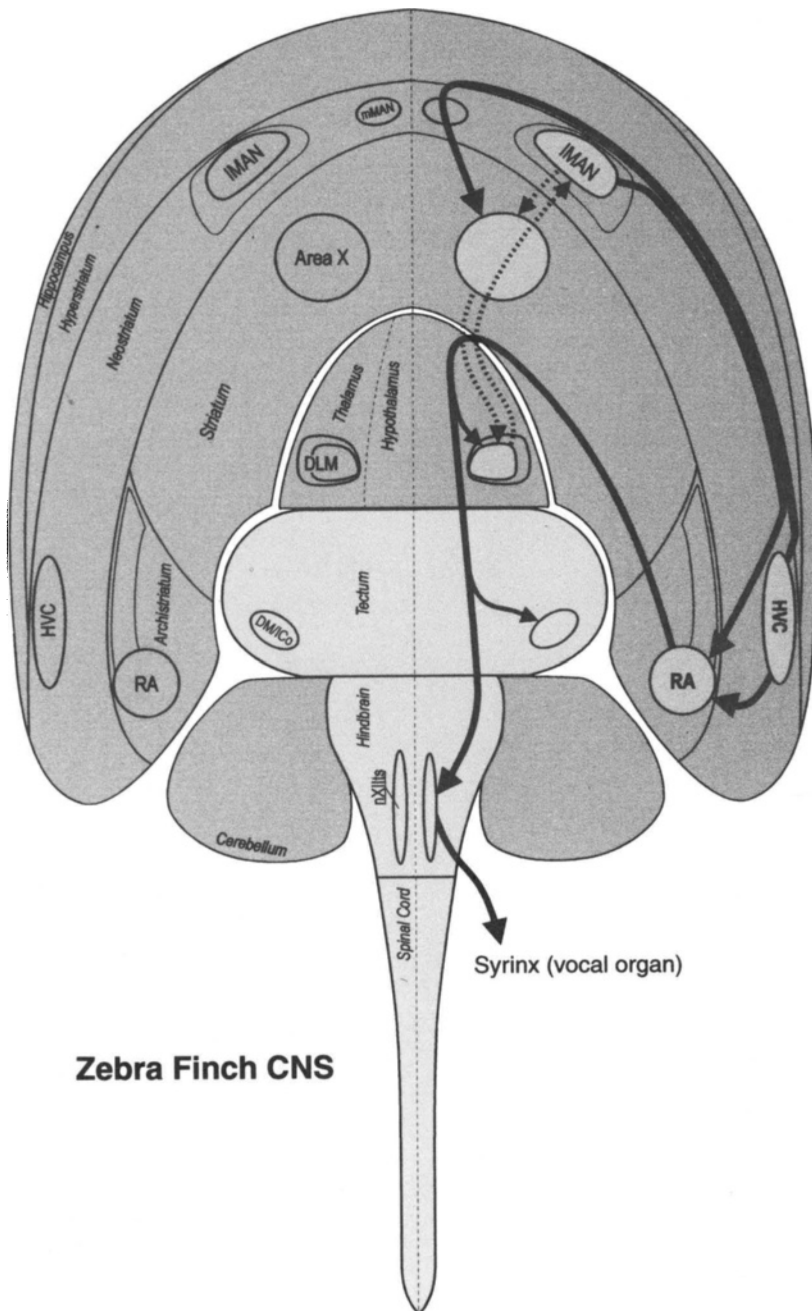

Figure 2. Flatmap of zebra finch CNS showing major song regions and their axonal connections. For the purposes of the present chapter, note that RA projection neurons comprise the major output of song-related processing in telencephalon. Abbreviations: Area X, Area X of Striatum; DLM, medial portion of the dorsolateral nucleus of the Thalamus; DM/ICo, dorsomedial nucleus of the Intercollicular Complex; HVC, Higher Vocal Center; lMAN, lateral magnocellular nucleus of the anterior Neostriatum; mMAN, medial magnocellular nucleus of the anterior Neostriatum; RA, robust nucleus of the Archistriatum; nXIIts, hypoglossal nucleus, tracheosyringeal portion.

phase of song learning is played out. Thus, by adulthood, song regions in vocalizing males are much larger and contain many more neurons, whereas non-singing females have lost many song control neurons.

The development of RA is perhaps most illustrative of sex-specific neurodegeneration in the song system (see Fig. 3). In males, RA volume doubles during the phase of vocal learning, an increase attributable to increased spacing between a fixed number of neurons and the addition of glial cells (Bottjer *et al.*, 1985; Bottjer *et al.*, 1986; Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989; Johnson and Bottjer, 1994; Nordeen and Nordeen, 1996). In contrast, female RA is roughly monomorphic to male RA at the time song learning begins, but then nearly half of RA neurons die, the surviving neurons decrease their density (i.e., move closer together), and the overall volume of female RA diminishes significantly (Bottjer *et al.*, 1985; Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989). Interestingly, despite the importance of auditory experience for song learning, deafening of juvenile males does not interfere with the normal development of the song system, at least in terms of volume and neuron number of song nuclei (Burek *et al.*, 1991).

3. UNCLEAR ROLE FOR GONADAL HORMONES IN SEXUAL DIFFERENTIATION OF THE SONG SYSTEM

Since the discovery that many neural song structures are sexually dimorphic (Nottebohm and Arnold, 1976), this system has remained one of the most dramatic exam-

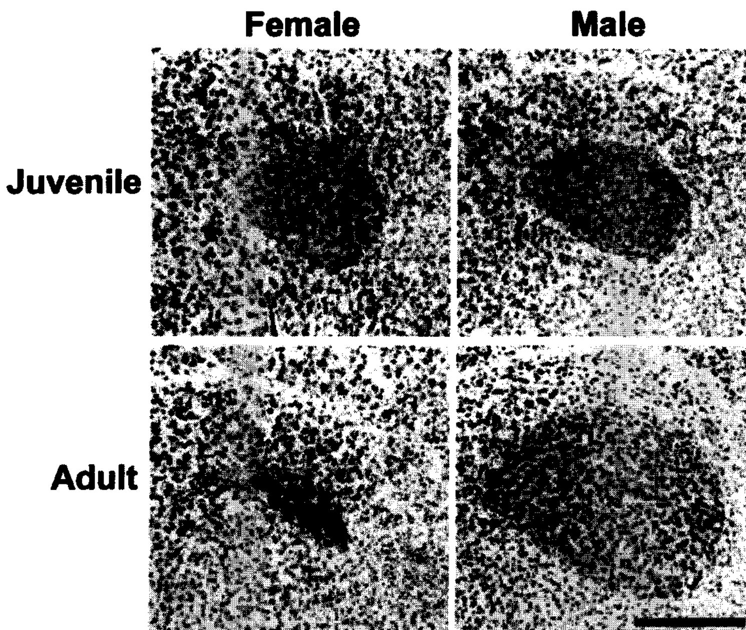

Figure 3. The development of RA in females and male zebra finches. At the beginning of the phase of song learning (~20 days of age), RA is roughly monomorphic in males and females. As song learning plays out, male RA doubles in volume, due to increased spacing between neurons (no net gain in neuron number). However, during the same developmental period in female, around 30–50% of RA neurons die and the remaining neurons pack closely together. Thionin-stained tissue, scale bar = 400 µm.

ples of a sex difference in forebrain structure. However, over 20 years later, we still do not fully understand the nature of the factors that produce such extreme differences in morphology during development. For example, one might expect gonadal steroids to be involved. A standard view is that exposure to androgens and/or estrogens drive male development, while low (or absent) levels of hormone lead to female development. Nevertheless, repeated attempts to sex-reverse developing male and female zebra finches with hormones have met with mixed results.

Estrogens appear to be important in that they can partially masculinze developing females (Gurney, 1981; Simpson and Vicario, 1991), but drugs that block estrogen synthesis fail to feminize males (Bottjer and Hewer, 1992). An additional problem for an estrogen hypothesis is that relatively few estrogen-sensitive cells are found in or near song-control regions (Nordeen *et al.*, 1987; Gahr and Konishi, 1988; Johnson and Bottjer, 1995; Jacobs *et al.*, 1996). One might think that androgens would be a good candidate for regulating sexual differentiation since many song regions contain neurons with androgen receptors (lMAN, HVC, RA, nXIIts; Arnold *et al.*, 1976; Balthazart, 1992; Johnson and Bottjer, 1993a). However, androgens are ineffective at masculinizing females and castration or blocking androgens only slightly impairs neural and behavioral development in males (Arnold, 1975; Bottjer and Hewer, 1992; Jacobs *et al.*, 1995). Recently, the very idea that hormones play a significant role in the sexual differentiation of the song system has been called into question. In a remarkable experiment, the gonads of embryonic female zebra finches were sex-reversed and although these birds developed true, functional testes and showed masculinization of some peripheral structures, their song-control systems remained essentially female (Wade *et al.*, 1996). These data may help explain why the many previous attempts to sex reverse the song system with hormones produced such puzzling results. Clearly, the striking growth of song regions in males, and the degeneration of song regions in females, are regulated by factors other than testicular hormones alone (Arnold, 1996).

4. EVIDENCE THAT CELL-CELL INTERACTIONS REGULATE SONG SYSTEM DEVELOPMENT

As elegantly detailed in previous chapters by Oppenheim, Parks, and Hyson, much of neural cell development in a variety of brain regions appears to occur via local signaling interactions between developing cells (e.g, release of a trophic factor from presynaptic cell to postsynaptic cell or vice versa). Numerous developmental parameters appear to be influenced by trophic factors, such as the formation of specific patterns of axonal connectivity, neurotransmitter phenotype, and the number of developing neurons that survive to adulthood. Applied to the avian song system, a "trophic factor" approach suggests that sex differences in the growth/degeneration of song control brain regions might be regulated by sex differences in the availability of trophic signaling molecules sent and received by developing song-control neurons (Johnson *et al.*, 1997).

We have recently begun to investigate the possibility that cell-cell signaling molecules (released pre- or post-synaptically or in an auto/paracrine fashion) regulate the sex-specific development of song-control regions. Since many song control brain regions receive relatively few sources of afferent input and make only one or two efferent projections (see Fig. 2), experiments to analyze the role of synaptic input or efferent targets on neural development are straightforward. For example, by making electrolytic lesions of lMAN in juvenile zebra finches, we have been able to examine the potential contribution of lMAN to the development of neurons that project to lMAN (the thalamic

nucleus DLM; Johnson and Bottjer, 1993b) and neurons that receive synaptic input from lMAN (the telencephalic nucleus RA; Johnson and Bottjer, 1994). Other investigators have employed a similar approach (Akutagawa and Konishi, 1994; Burek *et al.*, 1995) and together the results of these studies suggest that cell-cell interactions may play a significant role in the development of song-control brain regions, particularly with respect to the regulation of neuron survival during certain phases of neural and behavioral development.

4.1. Deafferentation of RA Induces Apoptosis

Our efforts to identify mechanisms by which developing song neurons influence one another's survival have focused on RA. RA receives two major sources of afferent input (from HVC and lMAN) and contains neurons whose axons leave the telencephalon and project to the brainstem motoneurons (nXIIts) that control the vocal organ (see Fig. 2). We and others have shown that removal of lMAN synaptic input to RA in fledgling birds (i.e., birds 20 days of age) induces the death of 30–50% of RA neurons (Johnson and Bottjer, 1994; Akutagawa and Konishi, 1994; Johnson *et al.*, 1997). Much of the deafferentation-induced RA neuron death occurs rapidly and synchronously (Johnson *et al.*, 1997). That is, at 24 hr post-deafferentation, the increase in the number of dying neurons in RA roughly matches the decrease in the number of RA neurons (see Fig. 4). This reduction in RA neuron number induced by deafferentation appears to be permanent but

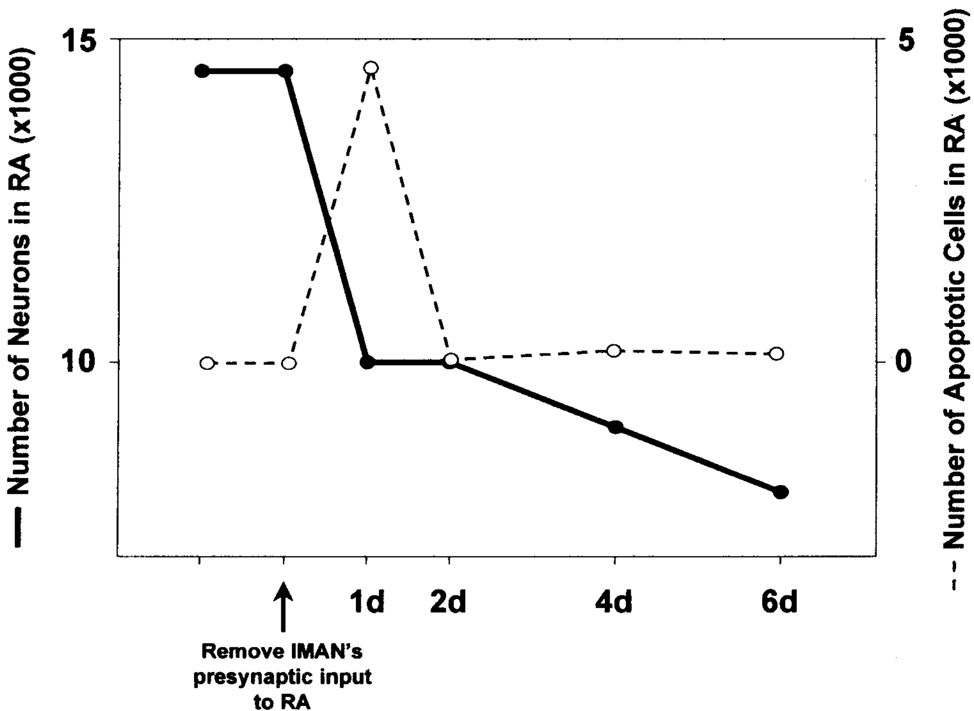

Figure 4. Synchronous apoptosis induced by deafferentation of RA in 20 d/o male zebra finches. One day after removal of lMAN presynaptic input to RA, a spike of apoptosis occurs in concert with an equally substantial loss of RA neurons. A more gradual loss of RA neurons appears to continue at 4 and 6 days post-lesion. This graph is a composite of data sets from Johnson and Bottjer (1994) and Johnson *et al.* (1997).

not progressive; if fledgling birds are given lMAN lesions and then survive to adulthood (90 days of age), their RAs contain 30–50% fewer neurons than normal adult males (Johnson, 1998).

The ability of lMAN lesions to induce neuron death in RA declines as birds mature. Birds that are in mid or late stages of vocal learning (40–60 days of age) or adult show no loss of RA neurons following lMAN lesions (Johnson and Bottjer, 1994). Thus, lMAN lesions induce RA neuron death during the developmental period that corresponds to the normal growth of male RA, and the normal loss of neurons in female RA (Kirn and DeVoogd, 1989; Johnson and Bottjer, 1994). These observations lead to the hypothesis that lMAN afferent input could influence the sex-dependent loss of neurons in RA. Interestingly, lMAN afferent input to RA shows a pattern of development that is consistent with this hypothesis. While both male and female lMAN contain the same number of RA-projecting neurons at the beginning of vocal learning (when RA is monomorphic between the sexes), the majority of RA-projecting neurons in lMAN are lost in developing females but are retained in developing males (Nordeen *et al.*, 1992). Thus, the naturally occurring loss of lMAN afferent input to RA in females could lead to the RA neuron death seen normally in females.

4.2. A Role for Neurotrophins in Song System Development?

The apparent dependence of RA neurons on synaptic input from lMAN suggests that lMAN neurons deliver a molecule to RA neurons that somehow promotes the survival of the latter. lMAN neurons could exert such an effect either by anterogradely transporting and releasing a neurotrophic factor directly onto RA neurons, or by influencing the auto/paracrine release of a neurotrophic factor by neurons or glia within RA. To determine whether neurotrophin growth factors play a role in afferent regulation of RA neuron survival, members of the neurotrophin family (NGF, BDNF, NT-3, NT-4/5) were tested for the ability to prevent the death of RA neurons.

The design of this experiment took advantage of the rapid time course of deafferentation-induced neuron death in RA (see Fig. 4). Lesions of lMAN were made bilaterally and neurotrophins were infused unilaterally into RA. Control (vehicle) infusions were made contralateral to the neurotrophin-infused RA. Comparisons of neuron number in neurotrophin-treated and control RA at 24 hr post-deafferetation showed whether the neurotrophin had death-preventative effects.

Infusions of BDNF, NT-3, or NT-4/5 into RA (1 μg doses at 0 and 12 hr post-lesion) all enhanced RA neuronal survival 24 hr following the removal of input from lMAN (Johnson *et al.*, 1997). Higher doses (2.5 μg) of BDNF and NT-3 produced a complete rescue of RA neuron number at 24 hr post-lesion (i.e., the number of neurons in deafferented RA was not different from that normally present in RA). In each instance where a neurotrophin dose preserved neuron number in RA there was also a clear suppression of apoptotic cells. That is, treatment with BDNF, NT-3, or NT-4/5 significantly reduced (or abolished, in the case of the 2.5 ug doses of BDNF and NT-3) the appearance of apoptotic cells in deafferented RA. Thus, the increased number of neurons in RA treated with various doses of BDNF, NT-3, or NT-4/5 is directly attributable to the ability of these neurotrophins to suppress neuronal apoptosis, as opposed to a neurotrophin effect on cell proliferation or migration. In contrast, infusions of NGF failed to suppress the appearance of apoptotic cells in RA and also failed to rescue RA neurons (Johnson *et al.*, 1997).

The above data suggest that lMAN could regulate the survival of neurons in devel-

oping RA by anterogradely transporting and releasing a neurotrophin, or by regulating the auto or paracrine release of neurotrophins within RA. However, to what extent does our demonstration that BDNF, NT-3, and NT-4/5 prevent induced neuron death reflect the normal state of affairs in developing RA? One place to begin is to ask whether the receptors for neurotrophins are present, and whether the expression of neurotrophins is consistent with a normal role in RA development.

Neurotrophins exert their cellular effects by binding to specific members of a family of receptors, the Trk family (TrkA, TrkB, TrkC; reviewed by Bothwell, 1995). For example, NGF binds with high affinity to TrkA, while BDNF and NT-4 bind with high affinity to TrkB. NT-3 is somewhat non-selective in that it binds with high affinity to TrkA, TrkB and TrkC, although TrkC is generally considered to be the NT-3 receptor in vivo. Based on the results of the neurotrophin infusion experiments (i.e., BDNF, NT-3, and NT-4/5 were effective, NGF was not), one prediction was that RA should contain TrkB and possibly TrkC receptors. That is, each of the three effective neurotrophins (BDNF, NT-3, NT-4/5) have the ability to bind with high affinity to TrkB receptors, while NT-3 might also exert an effect via high affinity binding to TrkC receptors.

When reacted with antibody against TrkB, RA showed heavy staining of fibers, neuropil, and somata throughout. In fact, TrkB staining was found throughout the telencephalon at high levels, similar to that observed in RA. Antibody staining against TrkC, the high affinity NT-3 receptor, revealed no soma labeling in RA, but sparse fiber labeling was found throughout the nucleus. Thus, the presence of TrkB and TrkC immunoreactivity in RA stands in good agreement with the infusion results, which showed that BDNF, NT-3, and NT-4/5 (i.e., neurotrophins able to bind with high affinity to TrkB, TrkC, or both) were able to maintain neuron number in deafferented RA (Johnson *et al.*, 1997). In contrast, an antibody against TrkA, the NGF high-affinity receptor, revealed no labeling within RA, perhaps explaining the inability of NGF to prevent RA neuron death. The low-affinity neurotrophin receptor, p75, was also detected immunohistochemically in RA and the telencephalon generally.

While neurotrophin receptor expression is consistent with results of the infusion experiments, the pattern of neurotrophin expression is more complex. Antibody labeling against NT-3 revealed immunoreactive neuronal somata throughout lMAN and RA—no staining of fibers or neuropil was evident (Johnson *et al.*, 1997). These data suggest that within lMAN *or* RA, NT-3 could be available for uptake and retrograde transport by a presynaptic population, auto/paracrine release, or anterograde transport and release. Given these results, NT-3 could be playing numerous developmental roles in lMAN and RA, in addition to a possible role in regulating RA neuron survival.

Antibody labeling against BDNF produced a more restricted pattern of labeling that changed as a function of development. That is, BDNF-immunoreactive somata are found in lMAN of 15–20 day-old birds (Johnson *et al.*, 1997), but BDNF labeled cells cannot be detected in lMAN of older juvenile birds or adults (Norstrom and Johnson, unpublished data). Thus, BDNF expressed by lMAN cells could be available for retrograde transport by a presynaptic population, auto/paracrine release, or anterograde transport and release to RA, but apparently only during a very early period of development. Interestingly, no BDNF labeling of somata is observed in RA at any stage of development, a contrast to the presence of TrkB receptors in RA. Thus, RA could conceivably respond to BDNF that is anterogradely transported and released from lMAN or HVC axons (see Fig. 2), whereas auto/paracrine BDNF release within RA seems unlikely.

It is worth noting that while the antibodies used for Trk receptor labeling are raised against avian Trks (Lefcourt *et al.*, 1996), the antibodies used for neurotrophin labeling

were raised against mammalian neurotrophins (Zhou and Rush, 1994; Yan *et al.*, 1997). Perhaps a clearer picture of neurotrophin expression in the developing song system will emerge as avian specific probes for neurotrophins are developed (also note that the expression pattern of NT-4/5 is not yet determined). Moreover, despite the preceding focus on neurotrophins and RA neuron survival, it should be emphasized that the development and sexual differentiation of RA entails a complex assortment of changes, involving the size of the nucleus itself, changes in the spacing and size of neuronal somata, and changes in the pattern of afferent input (Bottjer *et al.*, 1985; Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989; Johnson and Bottjer, 1994; Akutagawa and Konishi, 1994). Thus, it seems likely that more than one signaling molecule or pathway will be involved in mediating such changes. Recent studies suggest that non-neuronal (glial) factors (Nordeen and Nordeen, 1996) and retrograde influences (from the efferent targets of RA, Burek *et al.*, 1995) may be involved in regulating the sexual differentiation and development of RA. Finally, the role of activity in regulating RA neuron survival could also be important—chapters in this volume by Hyson and Parks clearly show that neurotransmitters themselves can be powerful regulators of neuronal development and survival.

5. MECHANISMS OF DEAFFERENTATION-INDUCED NEURONAL APOPTOSIS

Although there is accumulating information about the sequence of gene expression during neuronal cell death, the cytoplasmic mechanisms actually used by cells to kill themselves (i.e., undergo apoptosis) remain poorly understood. Because the death of RA neurons following lMAN lesions is rapid and massive, this is a system in which an extremely large population of dying neurons (~5,000) can be visualized and processed for various markers of cell function. Thus, utilizing an antibody against ribosomal RNA (Y10B, to assay levels of protein synthesis, see Hyson, this volume) and cytochrome oxidase histochemistry (to assay levels of oxidative metabolism, see Wong-Riley, 1989), striking changes in the protein synthesis machinery and oxidative metabolism have been observed in the cytoplasm of RA neurons undergoing apoptosis at 18–24 hr post-deafferentation. For example, dying neurons in RA show a complete loss of immunohistochemical staining for Y10B, suggesting that their protein-synthetic machinery has been disrupted or turned off (Johnson *et al.*, 1995; see Fig. 5). By comparison, neurons within an intact RA, or RA neurons that survive deafferentation show strong staining for Y10B. This pattern of lost protein synthetic ability in dying telencephalic neurons is similar to that reported for deafferentation-induced neuron death in the chick auditory brainstem (see Hyson, this volume, and Garden *et al.*, 1994).

However, all changes associated with apoptosis in RA do not appear to be degenerative in nature. When processed for cytochrome oxidase (CO) histochemistry, dying RA neurons show intense CO staining (Johnson *et al.*, 1995). In fact, the cytoplasmic compartment of the dying neurons, barely visible in light-microscopic analysis of Y10B- or Nissl-stained tissue, is quite apparent in tissue processed for CO histochemistry (see Fig. 5). By comparison, neurons within intact RA, or RA neurons that survive deafferentation, show low levels of CO staining and are not discernible unless tissue is counterstained. Because cytochrome oxidase is typically localized within mitochondria (where it is primarily known for its role in the respiratory chain), these data suggest that an increase in mitochondrial number or function may be related to the death of RA neurons.

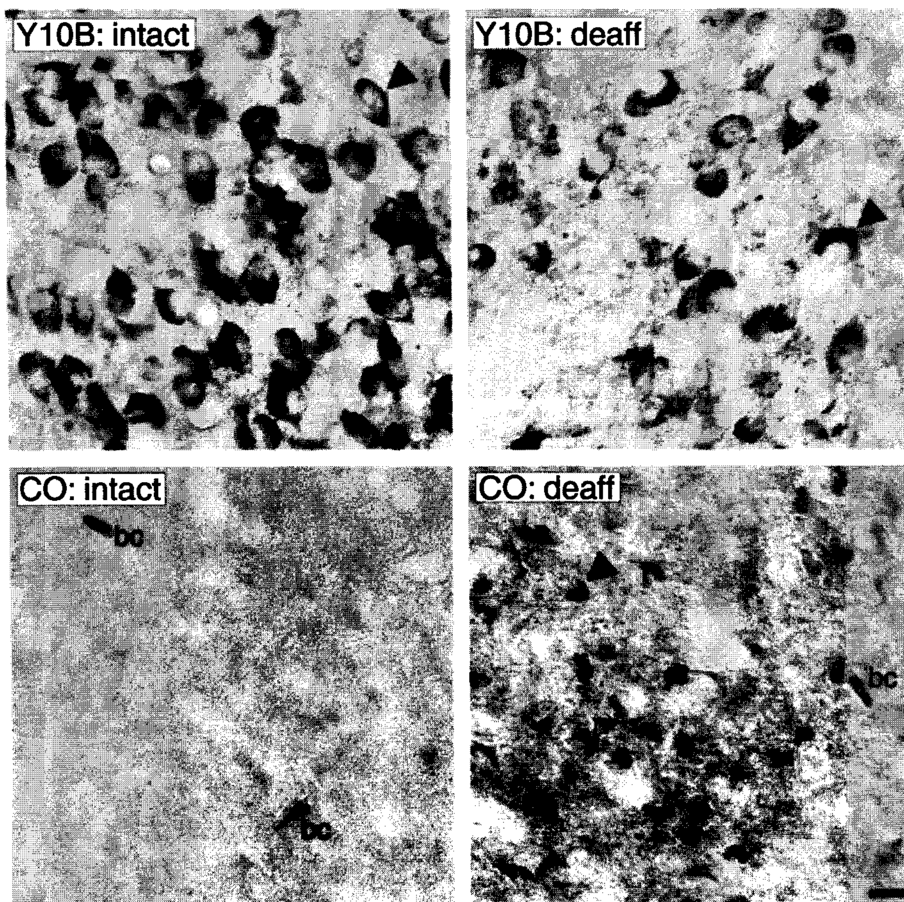

Figure 5. Dying RA neurons show contrasting protein synthetic and metabolic profiles. Normal RA neurons in 20 d/o males (left panels) show strong immunoreactivity for Y10B (an antibody against rRNA), while staining for cytochrome oxidase (CO, a mitochondrial enzyme) is low. By comparison, at 24 hr post-deafferentation there is a dramatic reduction in Y10B-labeled cells (Y10B: deaff), and a clear increase in CO-labeled cells (CO: deaff). Thionin counterstaining of deafferented RA (which labeled the pyknotic nuclei of dying cells) revealed that decreased Y10B labeling was due to a loss of Y10B immunoreactivity in dying RA cells, and that increased CO labeling was due to high levels of CO labeling in dying RA cells. Note that RA neurons condense considerably as they die—the size of the dying CO-labeled cells (CO: deaff, arrowhead) is much smaller than that of normal RA cells (Y10B: intact, arrowhead) and RA cells that survive deafferentation (Y10B: deaff, arrowhead). bc, blood cells; scale bar = 10 µm.

Interestingly, the low level of CO staining in RA neurons *surviving* deafferentation seems to differ from what has been observed in the chick auditory brainstem. Deafferented chick auditory brainstem neurons show a rapid, biphasic CO response; increased CO staining occurs in all neurons between 3 and 24 hr post-deafferentation, but levels dropped below baseline in surviving neurons at 3 days post-deafferentation (Hyde and Durham, 1990). Inhibiting mitochondrial proliferation (with chloramphenacol, a mitochondrial protein synthesis inhibitor) results in even greater levels of deafferentation-induced neuron death in the chick brainstem (Hyde and Durham, 1994), suggesting that a rapid upregulation of mitochondrial function is a key event in the ability of a neuron to survive deafferentation (Hartlage-Rubsamen and Rubel, 1996).

Thus, although a loss of protein synthetic function is apparently shared between brainstem and telencephalic forms of deafferentation-induced neuron death, the role of mitochondria may differ between these two forms of cell death. In the chick brainstem, an upregulation of mitochondrial activity may be necessary for neurons to survive deafferentation. In contrast, no change in CO staining is observed in RA neurons that survive deafferentation—an upregulation of CO occurs only in RA neurons that are dying. Increased CO levels in apoptotic RA neurons could represent a failed attempt to survive deafferentation, but it might also indicate that mitochondrial mechanisms participate in this form of cell death.

Mitochondria have been implicated in mechanisms of neuron survival, as well as mechanisms of neuron death. For example, excitotoxic neuron death seems to involve an overload of the calcium buffering function of mitochondria and treatments that increase/decrease mitochondrial depolarization have predictable effects on excitotoxic death (Schinder *et al.*, 1996; White and Reynolds, 1996). In this case, calcium buffering by mitochondria is an important pro-survival function. Alternatively, the production of oxide free radicals by mitochondria during cellular respiration has been implicated in "oxidative stress" models of disease- and aging-related neurodegeneration (Sims, 1996; Beal, 1996). Moreover, recent reports seem to show that the release of cytochrome-c from mitochondria is a triggering signal that leads to programmed cell death (Yang *et al.*, 1997; Kluck *et al.*, 1997). Determining whether mitochondria influence the life/death of RA neurons via pro-survival or pro-apoptotic mechanisms will be a goal of future research.

6. SUMMARY

The experiments and data presented in this chapter represent initial efforts to understand the development of the avian song control system in terms of cell-cell interactions. Our working hypothesis is that the sex-specific transformation of RA is at least partly influenced by the afferent input that RA receives from lMAN. This hypothesis is based on the observation that in juvenile birds, removal of lMAN afferent input rapidly induces the death of nearly half of RA neurons. Morever, degeneration of lMAN afferent input to RA is a normal developmental event in females, one that presumably leads to the normal developmental death of RA neurons in females.

A complete understanding of the mechanisms by which lMAN afferent input regulates the survival of RA neurons will require much future work, but the observation that neurotrophin infusions can rescue deafferented RA neurons (that would otherwise die) seems a promising start. Demonstrating that a neurotrophic mechanism underlies normal sexual differentiation, particularly the normal death of RA neurons in females, will require a more molecular level of analysis than has been employed to date. However, such work must also proceed with the awareness that multiple factors (e.g., other trophic molecules, neurotransmitters, and activity) are likely to be involved in orchestrating the overall development of RA.

Finally, an entirely unexpected outcome of our approach was the discovery that deafferentation of RA induced a rapid, synchronous wave of neuronal apoptosis, thereby paving the way for studies in which we can observe the mechanisms used by neurons to carry out a cell death program. To date this work suggests that apoptotic cells in RA undergo contrasting shifts in protein synthetic and metabolic function; a decrease in the former and an increase in the latter. An important next step is to determine whether one or both of these events are causal in the cell death process.

7. ACKNOWLEDGMENTS

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11

THE EFFECTS OF NEURONAL GROWTH AND SOCIAL EXPERIENCE ON THE DEVELOPMENT OF BEHAVIORAL PLASTICITY

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1. INTRODUCTION

The nervous system of a young animal experiences three kinds of plasticity as it grows: developmental changes, changes resulting from learning and memory, and experience-dependent changes in behavioral state. The first two are familiar and intertwined: neurons grow in size and complexity as sensory, motor and cognitive experience refine the synaptic organization of the brain during certain critical periods. At the same time, processes of learning and memory occur in and around synapses to enable sensory and motor experiences to be abstracted, categorized, and stored for later recall. Changes related to behavioral state are less studied at the level of neural mechanisms, but are also the subject of common experience. The behavior of all social animals is strongly conditioned by their social experience: their size and skill relative to their siblings and peers, or the social rank of their parents determines how they behave in the group. Moreover, sudden shifts in experience can lead to equally sudden changes in social behavior that may be short-lived or last a lifetime: the injury, disappearance or death of a dominant rival, or the appearance of a new, larger rival, can instantly and persistently change an animal's behavior.

As they grow, crayfish experience all three forms of plasticity. These plastic changes are apparent in the tailflip escape response, a rapid locomotor behavior performed by both small and large crayfish to escape from attack by rivals or predators (Wine, Krasne, 1982). The tailflip escape response is subject both to use-dependent habituation and to social experience-dependent modulation. Habituation, a form of non-associative learning, results from homosynaptic depression that occurs at synapses between primary sensory afferents and the set of mechansensory interneurons which they excite (Krasne, 1969; Zucker, 1972). Although homosynaptic depression is evident in the smallest free-swimming juveniles, behavioral habituation is absent, and only appears as the animal and

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the neurons of the tailflip circuit grow to a critical size (Fricke, 1984; Edwards *et al.*, 1994). Modulation of the tailflip escape circuit is also evident in the smallest animals, where serotonin can change the stimulus threshold of the circuit. The nature and sign of this modulation depends, however, on the early social experience of the animal, so that it can facilitate escape in socially isolated and socially dominant individuals, and inhibit escape in socially subordinate animals (Yeh *et al.*, 1997; Yeh *et al.*, 1996).

2. TAILFLIP ESCAPE BEHAVIOR IN JUVENILE CRAYFISH

Tailflip escape behavior is relatively more important in the lives of juvenile crayfish than in the lives of adults because of differences in their abilities to defend themselves. After hatching from eggs attached to their mother's swimmerets underneath her abdomen, crayfish fry cling to the same swimmerets until after their second molt, when they drop off to lead free-swimming, independent lives (Fig. 1) (Huxley, 1880). At this time, the juvenile animal resembles the adult, except that its abdomen is relatively larger, and the claws (chelipeds) are relatively smaller. These differences, plus the much thinner and more fragile exoskeleton, lead the young crayfish to be more dependent on tailflip escape to defend from attacks by conspecifics or predators. Whereas adult crayfish will use their large claws and heavy armor to defend against attack, the primary defense of juvenile crayfish is the tailflip response, which is a rapid flexion and re-extension of the abdomen that carries the animal away from the attack (Fig. 2).

There are three forms of tailflip that differ in the nature of the trigger stimulus, the neural circuit that produces the response, and the form and direction of the tailflip (Wine, Krasne, 1982). "Lateral Giant" (LG) tailflips are excited by attacks on the rear of the animal, and lead to somersault tailflips that pitch the animal up and forward off the substrate. LG tailflips result from activation of the LG neuron by massive convergence of sensory inputs excited by the attack on the rear of the animal. The LG is a "command neuron" for tailflip: a single spike in the LG is both necessary and sufficient to trigger a tailflip (Olson, Krasne, 1981). The LG tailflip is a very short-latency (~25 ms) flexion of the abdomen around the thoracic-abdominal joint, while the rest of the abdomen is held rigid. This pitches the animal up and forward (Fig. 2). The initial fast abdominal flexion is followed by re-extension, and then by a series of quick cycles of flexion and extension that constitute swimming (Reichert, Wine, 1983; Reichert, Wine, 1982; Reichert *et al.*, 1981; Krasne, Wine, 1984; Krasne, Wine, 1987).

An equally sudden attack on the front of the animal will produce a similarly massive convergence of sensory inputs on another command neuron, the medial giant (MG) interneuron (Wine, Krasne, 1972). The MG is connected to many of the same pre-motor interneurons and motorneurons as LG, but in a different segmental pattern that causes the abdomen to flex around all the abdominal joints. This promotes a rapid curling of the abdomen that throws the animal backward with a similar short latency (Fig. 2). As with the LG response, an MG tailflip is followed by rapid re-extension and swimming.

The third type of tailflip is either voluntary or is evoked by less sudden stimuli, and, unlike the highly stereotyped LG and MG taiflips, produces a long-latency (200–300 ms) tailflip that is directed away from the stimulus. Such "non-giant" tailflips are produced by a poorly described network of interneurons that activate the set of segmental abdominal motorneurons in a pattern appropriate for moving the animal in the desired direction (Reichert, Wine, 1983).

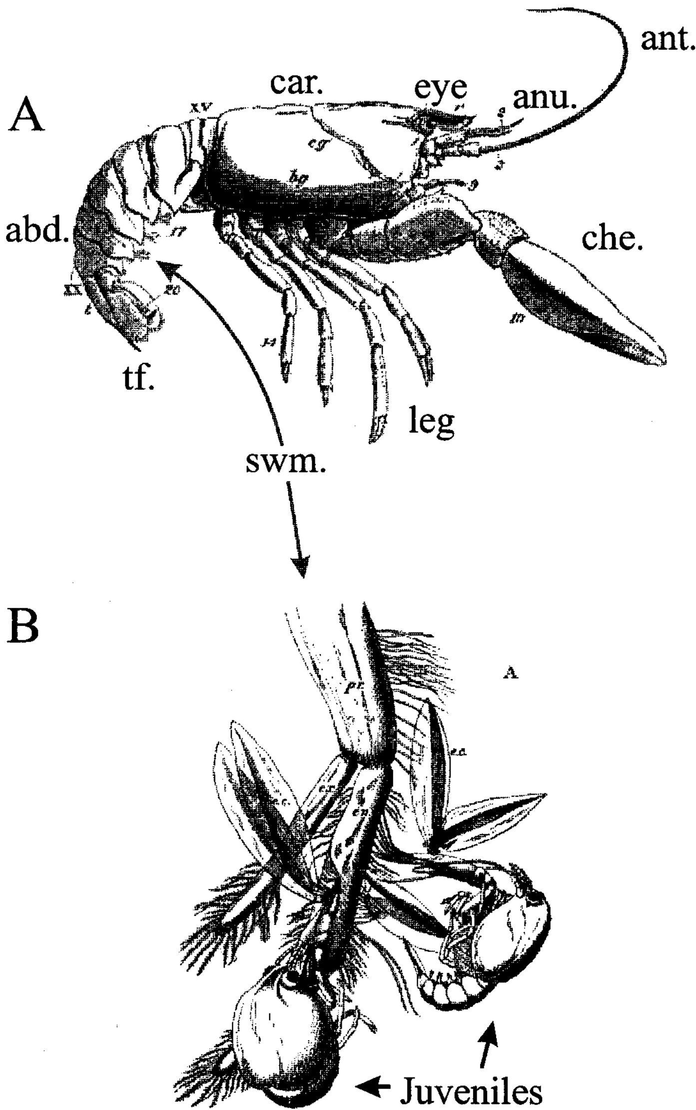

Figure 1. Adult and juvenile crayfish (from Huxley, 1880). A. Lateral view of an adult crayfish, showing the (ant.) antennae, (anu.) antennules (the "nose"), the (eye) eyestalks and compound eyes, the (che.) chelipeds or major claws, the (leg) walking legs, the (car.) carapace, the (abd) segmented abdomen, the (swm.) swimmerets, and the (tf.) tailfan. B. An expanded view of a pair of juvenile crayfish hanging on by their claws to one of their mother's swimmerets. The arrow identifies the location of the swimmeret on the adult animal.

3. THE LG TAILFLIP ESCAPE CIRCUIT

The circuit that mediates the LG tailflip is one of the best understood neural circuits in any animal (Edwards *et al.*, 1999); the afferent portion is illustrated in Fig. 3. Sensory afferents converge on the LG neuron and other mechanosensory interneurons (MSIs) from hairs that cover the abdomen and from stretch receptors in the joints of the tailfan (Nagayama *et al.*, 1997; Newland *et al.*, 1997; Zucker, 1972). One population of

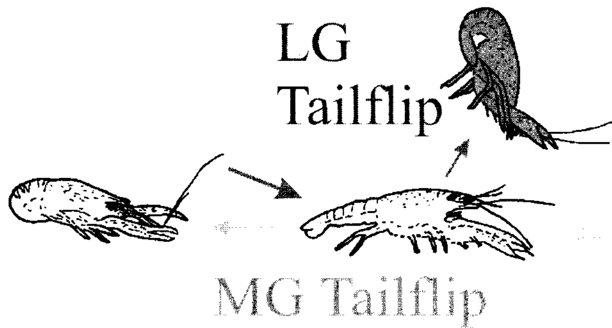

Figure 2. Two forms of tailflip. The LG tailflip (darker drawing above) occurs in response to a stimulus directed at the tail of the resting animal (darker arrow directed at abdomen of clear drawing of extended crayfish); the MG tailflip (lighter drawing behind) occurs in response to a similar phasic stimulus directed at the front of the animal (lighter arrow to front of the extended animal).

afferents contacts the LG directly through rectifying electrical synapses, whereas others contact MSIs through nicotinic cholinergic synapses (Lee, Krasne, 1993; Edwards *et al.*, 1991). These interneurons then contact LG through rectifying electrical synapses, so that all of LG's excitatory inputs are made through these voltage-sensitive electrical synapses (Edwards *et al.*, 1998). The monosynaptic contacts from primary afferents produce an early, "α" wave of depolarization in LG that is quickly succeeded by a "β" wave produced by inputs from the MSIs.

The afferent synapses from both primary sensory cells and MSIs are made on the distal portion of LG's major dendritic branches (Lee, Krasne, 1993). Compound EPSPs in these dendrites can achieve very large amplitudes—80 mV or more—but these are

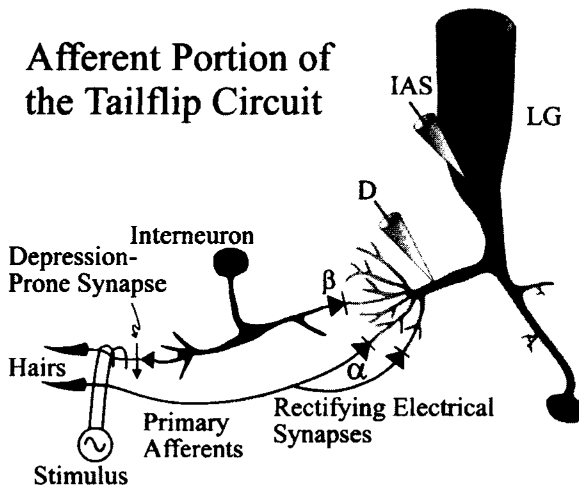

Figure 3. The afferent portion of the LG escape circuit and the experimental arrangement. One or more sensory nerves is electrically shocked (Stimulus) to excite the Primary Afferents and Interneurons that make Rectifying Electrical Synapses with LG. Depression-Prone Synapses occur between the primary afferents and interneurons. Inputs to LG from the primary afferents give rise to the α component of the compound EPSP, and inputs from the interneurons produce the β component. Recordings were made from the proximal portion of the LG dendrite (D) and from the initial axon segment (IAS).

rapidly attenuated as they conduct passively to the initial axon segment where spikes are initiated (Edwards *et al.*, 1994b; Kennedy *et al.*, 1971b). There the EPSPs rarely exceed 10 mV.

A rapid tap on the abdomen, or a shock of one or more sensory nerves will excite mechanosensory afferents and interneurons, and they, in turn, will excite LG to produce a single spike. Several mechanisms prevent repetitive firing, which, in view of the sufficiency of one LG spike to launch a tailflip, would be maladaptive (Vu *et al.*, 1997; Roberts, 1968).

4. DEVELOPMENT OF TAILFLIP HABITUATION

The strength of a tap necessary to evoke a tailflip varies with the size of the animal. The tap delivered by a small probe that is strong enough to evoke an LG-mediated tailflip in a 1 cm juvenile crayfish will be inadequate to excite a 2 cm animal; correspondingly larger stimuli are needed for larger crayfish. The stimulus threshold for tailflip increases with size of the animal despite the corresponding increase in the size of the tailfan and the number of primary afferents that contact LG. Very large (>12 cm crayfish) will tailflip only in response to the strongest, sharpest attacks; they prefer to stand with chelipeds raised in defense instead.

In addition to an increased stimulus threshold for tailflip, growth also brings the onset of response habituation. Repeated taps will evoke LG spikes and tailflips from adult animals on the first few trials, but not thereafter (Krasne, 1969). Habituation of the tailflip response does not occur in very small animals; repeated taps on the abdomen of 1 cm juvenile crayfish reliably evoke tailflip escapes triggered by LG (Fricke, 1984; Edwards *et al.*, 1994). Habituation first appears when the animal is between 2 and 3 cm long, in response to repeated stimuli that are initially just superthreshold. These animals will still tailflip reliably to stronger stimuli. As the animal grows larger, habituation becomes more pronounced, and will develop even to the strongest stimuli.

This habituation has two sources, one extrinsic to the circuit, and one intrinsic to it. Descending tonic inhibition of the LG, which increases with repeated stimulation, produces the extrinsic habituation (Krasne, Teshiba, 1995). The intrinsic habituation derives from homosynaptic depression at the nicotinic cholinergic synapses between the primary sensory afferents and the MSIs (Fig. 3); synaptic transmission (i.e., quantal content) decreases with repeated activation, so that fewer MSIs reach threshold in response to later taps. As a result, LG receives synchronous inputs from fewer MSIs, and so produces a smaller β component of the compound EPSP (Zucker, 1972).

4.1. Unmasking Depression-Prone Synapses and the Onset of Habituation

What changes in the escape circuit to enable intrinsic habituation to develop as the animal grows to exceed 2.5 cm in length? One possibility was that the nicotinic cholinergic synapses between the primary afferents and MSIs changed from being reliable to being depression-prone, as occurs in *Aplysia* (Mercer *et al.*, 1991). A second possibility was that the monosynaptic pathway was sufficient to excite LG in small animals, but not in larger animals, when the disynaptic pathway containing the depression-prone synapse took over as trigger for LG.

Recordings from the proximal dendrite and the initial segment of LG in small juveniles, mid-sized animals, and adults show (i) that synaptic depression reduces the β EPSP

in all three sizes of animals, and (ii) that as the stimulus intensity is increased, LG is excited by the reliable monosynaptic α EPSP in small crayfish (Fig. 4), by either α or β in mid-sized animals, and by β alone in adults (Fig. 5). These results indicate that the depression-prone synapses are present in the youngest animals, but their effects are not expressed as behavioral habituation because the earlier α EPSP provides a reliable trigger for the LG and the tailflip response (Fig. 4) (Edwards *et al.*, 1994b; Edwards *et al.*, 1994b).

Habituation at the depression-prone synapse is unmasked in mid-sized animals (~2.5 cm). The α EPSP is smaller than the β EPSP in these animals, and low stimulus intensities can bring the β EPSP to threshold to fire LG (not shown). Repeated stimuli at these intensities cause the LG response to habituate as β depresses below threshold. Stronger stimuli will bring the earlier α EPSP to threshold, and LG's response assumes the reliable character of the α EPSP (Edwards *et al.*, 1994a).

In adults, the α EPSP is small enough so that it can usually not be brought to threshold at all, so that LG is triggered exclusively by the β EPSP. (Fig. 5). The LG response and tailflip behavior habituate to all levels of repetitive stimulation.

4.2. Failure of the α EPSP to Compensate for Growth

Two mechanisms contribute to the failure of the α EPSP to trigger LG's response as the cell grows: a decline in the relative strength of the α synaptic input, and increasing attenuation of the α EPSP between the dendritic input sites and the proximal spike initiation zone (Edwards *et al.*, 1994b). As LG grows, its input resistance decreases, so that a constant synaptic current will produce a smaller EPSP. The growth-related decline in input resistance is counteracted by the addition of hair cells to the abdominal surface during growth, so that the population of primary afferents that project to LG and to other MSIs increases. Despite this increase in primary afferent inputs, the strength of the monosynaptic input (α) declines relative to the depression-prone disynaptic input (β) during growth of the animal. This result is apparent in Fig. 4, where recordings in small animals show that the α and β EPSPs are comparable in amplitude, whereas in large animals (Fig. 5), α EPSPs in dendrites and the initial segment are consistently smaller than β EPSPs.

4.3. Growth Increases the Relative Attenuation of the α EPSP

The failure of the α EPSP to compensate for growth is compounded by an increase in the relative attenuation that the α EPSP experiences (Fig. 6). The compound EPSP is greatly attenuated as it spreads from the dendrites to the initial axon segment, and this attenuation increases with growth. In small animals, α and β EPSPs experience similar attenuation, but in adult animals, the α EPSP experiences much more attenuation than the β EPSP. As a result, the reliable α EPSP becomes less able to fire LG, whereas the depression-prone β EPSP remains able to do so. The difference in attenuation increases with growth until the reliable α EPSP fails to excite the LG, and the trigger passes to the later, and more labile β EPSP. At this point, the depression-prone characteristic of the β EPSP is unmasked as the LG tailflip habituates to repetitive stimulation (Edwards *et al.*, 1994b).

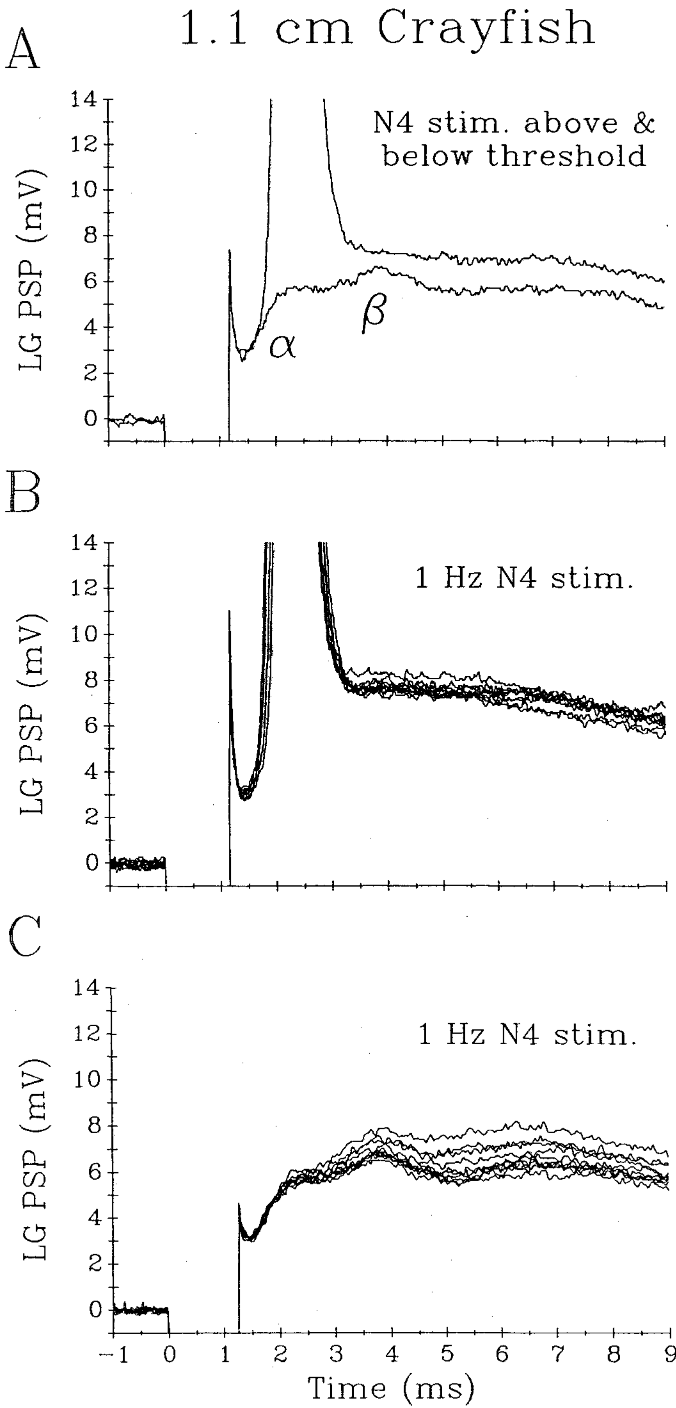

Figure 4. LG is triggered exclusively by the reliable α EPSP in a small juvenile crayfish. A. Below- and above-threshold responses of LG to single shocks of nerve 4 (N4) in the terminal ganglion. The α and β EPSP components are labeled. B. Superthreshold stimulation of N4 at 1 Hz. C. Subthreshold stimulation of N4 at 1 Hz. Note that β, but not α, depresses.

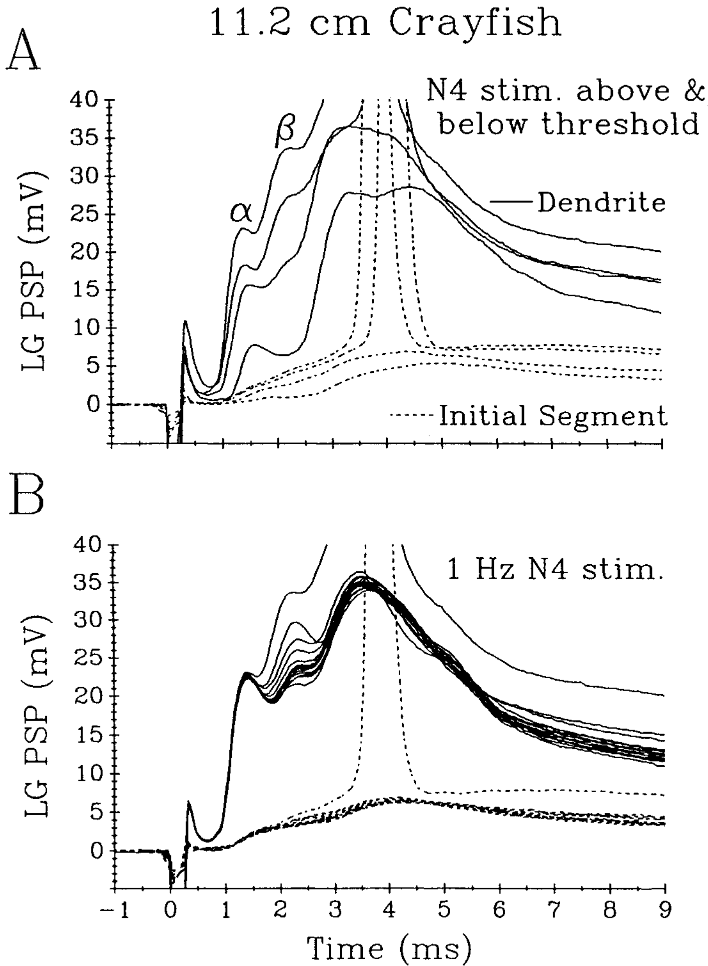

Figure 5. LG is triggered exclusively by the depression-prone β EPSP in an adult crayfish. A. LG responses to stimuli of different amplitudes recorded in the proximal dendrite (solid lines) and the initial axon segment (dashed lines). Note that α is always smaller than β. B. Repetitive stimulation of N4 at 1 Hz at the highest stimulus level in A. Note that the β EPSP habituates and falls below LG threshold while the α EPSP remains stable.

4.4. Isoelectrotonic Neuronal Growth

The change in attenuation results from the pattern of growth of LG: the dendrites and axon grow longer at a faster rate than they grow in diameter. If the density of membrane channels and synapses remains constant, this pattern of growth will lead to growth in the electrical structure of the neuron: each part will become electrically more distant from the others (Hill *et al.*, 1994; Edwards *et al.*, 1994). Mathematical analysis of the effects of different patterns of uniform neuronal growth on the electrical structure of neurons has shown that if the diameters of a neuron's processes (dendrites and axon) grow as the square of their increase in length, then the electrical structure of the neuron will not change: a voltage created at corresponding points in the small and large cell will create the same pattern of active and passive responses at all other corresponding points in the two cells (Olsen *et al.*, 1996). This pattern of "isoelectrotonic" growth will maintain the electrical structure of the neuron regardless of its initial shape or distribution of

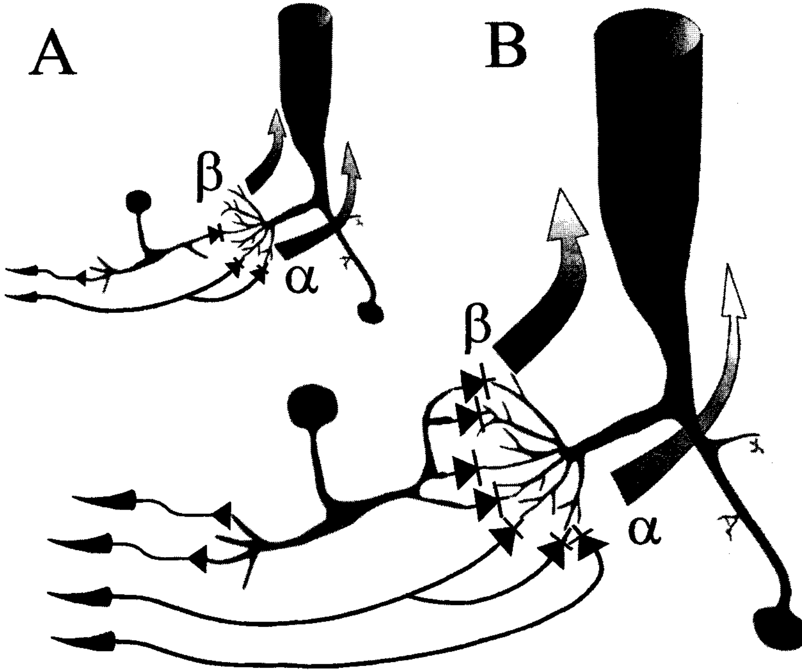

Figure 6. Diagram of the afferent circuit to LG in small (inset) and large crayfish. Growth of the animal is accompanied by growth of all the neurons, an increase in the number of hairs on the abdominal surface, and an increase in the number of primary afferents to LG and the MSIs. The ability of the MSI input to compensate for growth is illustrated by the addition of several synaptic inputs onto LG, whereas the failure of the primary afferent input to LG to compensate is illustrated by fewer additional synapses. The voltage attenuation experienced by EPSPs in the LGs of both animals is indicated by the curved arrows directed from the dendrites towards the initial segment. The increased relative attenuation experienced by the α EPSP in the adult crayfish is indicated by the shading and slimmer shape of the arrow from the primary afferent input to the initial segment.

membrane properties, so long as the local density of ion channels remains constant. Isoelectrotonic growth also defines a watershed between patterns of growth that lead to increased attenuation and those that lead to decreased attenuation. If, as with LG, the diameters of a neuron's dendrites grow at a rate less than the square of their increase in length, then the cell will become electrically larger, and if the diameters grow at a greater rate, the cell will become electrically smaller (Olsen *et al.*, 1996).

Nonuniform growth, in which different parts of a neuron follow different patterns of growth, is likely to be more common than uniform growth. Work with computer models of growing neurons has shown that different combinations of isoelectrotonic growth and isometric growth (i.e., when the diameter grows at the same rate as the length) can lead either to an increase or a decrease in the electrical size of a neuron.

These theoretical results show that the pattern of a neuron's growth must be actively controlled, not just to ensure that the correct synaptic contacts are made, but also to ensure that its integrative properties are well-adapted to the role that the neuron plays in its circuits. This may mean that the neuron should retain its original integrative properties despite increases in physical size, or that the neuron should become electrically larger or smaller.

5. EXPERIENCE-DEPENDENT PLASTICITY

Juvenile crayfish hatch together with up to 300 siblings in the chamber of a burrow from one to six feet below the surface. As they become free-swimming, they will leave the burrow if they are able. If the mouth of the burrow is under water, they will all leave quickly by swimming; if not, those that can will leave by walking out of the burrow through the mud, and the others will stay behind. Juvenile crayfish raised together in an aquarium will cannibalize each other, with the larger ones eating their smaller siblings, until only a few are left. It is likely that this is the fate of those animals that are unable to leave the brood chamber: the larger ones will eat the smaller ones until the water rises and they can swim out, or until the survivors are large enough to walk out.

In such crowded conditions, crayfish form dominance hierarchies (Bovbjerg, 1953). Studies of groups of 5 previously isolated juvenile crayfish grouped together in a small 18 cm x 18 cm square aquarium indicate that they form tyrannies, in which one animal is superdominant to the others (Issa *et al.*, 1999). Initially that animal makes nearly all of the attacks and the others escape or retreat. In the days following their first encounter, the frequency of the dominant's attacks on subordinates declines greatly, and are replaced on half the occasions with less vigorous approaches. In response, the subordinates choose to retreat before contact occurs rather than to escape by tailflipping, which becomes very infrequent.

5.1. The Effect of Social Status on the LG Escape Response

How is the nervous system, which mediates these behavior patterns, affected by the change in social status? The LG taifiip response is one of many behavior patterns so affected: experiments in freely behaving adult crayfish have shown that the stimulus threshold of the LG response increases in subordinate animals during fights, but not in dominant animals, where it may decline (Krasne *et al.*, 1997). At the same time, the threshold for non-giant (voluntary) tailflips falls in subordinate animals. These results are understandable when one remembers that the LG tailflip is evoked exclusively by external attacks on the rear of the animal. A subordinate animal in a fight with a more dominant crayfish will likely be moving backwards much of the time, and may well bump into a fixed object or another crayfish. An LG tailflip triggered by such a bump would throw the animal up in a somersault towards its opponent. Perhaps to avoid this and to enable the subordinate animal to pick the time and direction of its escape, the LG response is inhibited while the non-giant escape is facilitated. The dominant animal, which would tend to move forward during the fight, is not likely to trigger an LG tailflip inadvertently. As a result, the dominant may not inhibit its LG response, and may even facilitate it in circumstances when the animal risks attack by a third party.

5.2. Social Status Modulates the Effect of Serotonin on LG Threshold

Serotonin is a neuromodulatory substance that has been implicated in the mediation of social dominance and aggression across animal phyla, including lobsters and crayfish (Kravitz, 1988; Edwards, Kravitz, 1997). In crayfish, serotonin injected into a new subordinate animal promotes an unexpected and protracted aggressive response towards its dominant partner (Huber *et al.*, 1997). This effect, which can be blocked by injection of the serotonin uptake blocker fluoxetine, suggests that the release of endogenous serotonin may facilitate aggressive responses in crayfish during fights.

Serotonin also modulates the threshold of the LG tailflip escape response (Glanzman, Krasne, 1983). Recently it was found that serotonin's effect on LG depends on the social status and social history of the animal (Yeh *et al.*, 1997; Yeh *et al.*, 1996). Serotonin facilitated LG's response in juvenile crayfish that had been isolated for more than a month since becoming free swimming. In these animals, serotonin enhanced both α and β EPSPs and reduced the stimulus threshold for an LG spike. These effects persisted for several hours following removal of the applied serotonin with a saline wash.

When the socially isolated crayfish were paired in a small cage, they immediately fought and formed a dominant/subordinate pair. If this pair was kept together for 12 days, it was found that the effects of serotonin on LG had changed: It was inhibitory in the new subordinate and facilitatory in the new dominant, and in both animals its effects were readily removed with saline wash. These effects developed linearly over the 12 day period following pairing, and could be reversed if the paired animals were re-isolated for another 8 days (Fig. 7A).

Social promotion, but not social demotion, also led to a change in serotonin's effects. When social subordinates were paired, one of the former subordinates became dominant following an initial fight. The effect of serotonin in this animal changed linearly over the next 12 days from being inhibitory to being facilitatory, like the dominants that derived from pairing isolate crayfish (Fig. 7B). However, when social dominants were paired and one became subordinate, the effect of serotonin remained facilitatory even after 40 days of pairing. It is apparent then, that as in other animals, the physiological phenotype associated with social dominance is the preferred state (Fig. 7C).

5.3. The Population of Serotonin Receptors Changes with Social Status

How can the same substance, serotonin, have such different effects on the excitability of LG in animals that differ only in their social status? The simplest explanation is that the different animals possess different populations of serotonin receptors that, as in vertebrates, have opposing or different effects on a cell's excitability (Peroutka, 1988). Crustaceans have been found to have several different types of serotonin receptors, but these receptors do not appear to map onto existing classes of vertebrate receptors (Zhang, Harris-Warrick, 1994). Receptors that are activated by a class of specific vertebrate 5-HT (i.e., serotonin) receptor agonists are antagonized by substances that antagonize other vertebrate receptors.

The effects of two vertebrate agonists, α-Me-5-HT, a vertebrate 5-HT_2 receptor agonist, and piperazine, a 5-HT_1 receptor agonist, were tested for their effects on LG in isolate, subordinate and dominant crayfish (Yeh *et al.*, 1997; Yeh *et al.*, 1996). Piperazine had no effect on LG in isolates, but was strongly inhibitory in dominants and subordinates. These effects were removed by saline wash. The inhibitory effect of piperazine developed linearly in both dominant and subordinates over a 12 day period following pairing, and was reversed by re-isolation of the pairs. This time-course was nearly identical to the development of the inhibitory effect of serotonin in new subordinate crayfish.

α-Me-5-HT had the same effect on LG in social isolates as serotonin: EPSPs were enhanced and the cell's stimulus threshold was reduced, and remained reduced for several hours following removal of the drug by a saline wash. It had a similar effect in 12-day social dominants and subordinates: EPSPs were enhanced and stimulus thresholds were reduced, but these effects were quickly removed by a saline wash.

The results are consistent with the suggestion that two, and perhaps three different

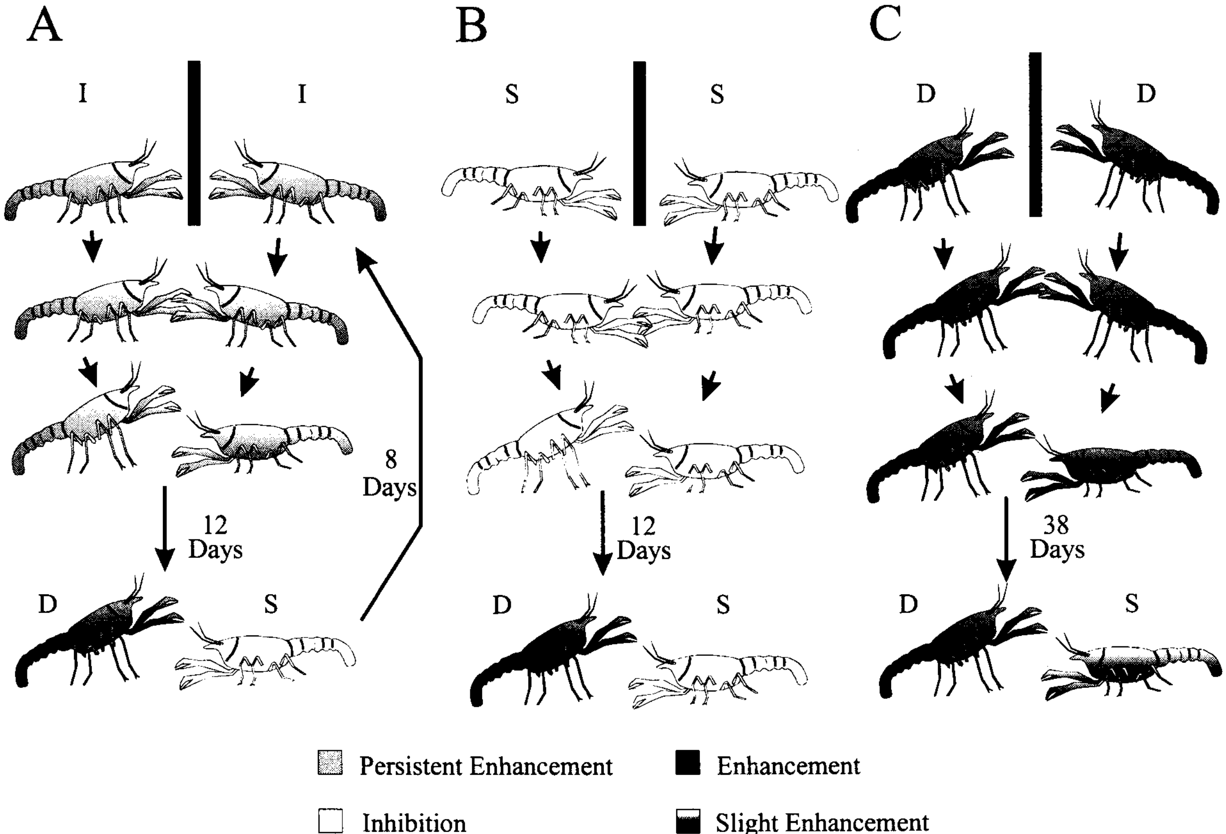

Figure 7. Illustration of the behavioral and physiological changes produced by pairing social isolates (I), social subordinates (S), and social dominants (D). The posture of the cartoon crayfish indicates the status of the animal: dominants are indicated by the elevated, upwards posture, subordinates are indicated by the lowered, flat posture, and isolates are indicated by the middle posture. The coloring of the cartoon represents the effect of serotonin on LG's response according to the legend at bottom. The vertical black bar represents a screen that initially keeps the animals apart. In each sequence, from top to bottom, the screen is pulled away (top), the animals fight (2nd row), one becomes dominant and the other subordinate (3rd row), some period of time passes as the effect of serotonin changes. A. Effect of pairing social isolates. The upward arrow on the right side of the panel indicates that when the dominant or subordinate animals were re-isolated, their physiology and behavior reverted to that of isolates. B. Effect of pairing social subordinates. C. Effect of pairing social dominants.

serotonin receptors mediate the effects of serotonin observed in isolate, subordinate and dominant crayfish. In socially isolate crayfish, a facilitatory receptor is present that has perisistent effects. Upon pairing, this receptor is either modified or replaced with a receptor that has facilitatory effects that depend on the presence of the agonist. This receptor is present in both dominant and subordinate crayfish. Pairing also promotes the appearance in both animals of an inhibitory receptor which has effects that also depend on the presence of the appropriate agonist. In dominant crayfish, the balance of effects favors the facilitatory receptor, whereas in subordinate crayfish, it favors the inhibitory receptor. Re-isolation or social promotion shifts the population of receptors again, and over the same 12-day timecourse; social demotion has little effect on the receptors.

5.4. Short- and Long-Term Adaptations to a Change in Social Status

The change in serotonin receptors on the LG neuron is just part of the adaptation to a change in social status. The immediate change in behavior following the critical victory or loss probably results from a change in the balance of neuromodulators that affect critical circuits in the nervous system. Whereas injected serotonin can promote aggression and a dominant postural stance, injected octopamine promotes a supine posture and submissive behavior (Antonsen, Paul, 1997; Livingstone *et al.*, 1980). It is likely that these neuromodulators are released endogenously during agonistic interactions to promote a pattern of behavior that is appropriate to an animal's status relative to its opponent. More concrete changes in the nervous system, including in the population of serotonin receptors, also begin to occur, but they appear to accrete slowly and only become apparent after several days experience in the new social order. This slow transition to a new receptor population ensures that such changes in the "hardware" of the nervous system will not be undertaken just because the animal has had a bad day (Yeh *et al.*, 1997).

Although the current social organization may last a lifetime, it may also end tomorrow if the dominant animal leaves or is disabled. Because dominant status is preferred, subordinate animals would do well to be prepared to take advantage of the opportunity to become dominant. Given this opportunity, the receptor population appears able to shift from the subordinate state to the dominant state over the same time-course that was necessary to shift from the isolate state to the subordinate state. Interestingly, the receptor population does not readily shift back following a loss of dominant status, perhaps because for many animals the likelihood of regaining dominance is high.

6. NEURAL PLASTICITY DURING POST-EMBRYONIC GROWTH

Both types of plasticity, synaptic depression at the first-order synapse, and status-dependent modulation of LG, are present in the youngest free-swimming animals. Synaptic depression is not expressed as habituation of LG excitability (or tailflip behavior) until the animal exceeds 2.5 cm, whereas status-dependent serotonergic modulation of LG is expressed in all animals tested (>1 cm). Presumably, LG and tailflip thresholds in freely behaving juvenile crayfish are modulated by aggression and social status in a manner similar to their modulation in adult animals. Consistent with this, studies of dominance hierarchies among groups of juvenile crayfish show that escape tailflip is one of several behaviors that vary in their frequency according to social dominance status (Issa *et al.*, 1998).

Why should habituation of the tailflip response (and its stimulus threshold) vary with the age or size of the animal, whereas status-dependent modulation of LG occurs in a similar fashion in all sizes of animals? A usual way of thinking about the relation of plasticity to growth is in terms of maturation, whereby the capabilities and flexibility of the nervous system and behavior increase with the maturity and experience of the animal. Perhaps more appropriate for these animals, however, is to think in terms of the requirements of the different niches the animal must occupy as it grows. A mother crayfish and her two hundred juvenile offspring differ by 20-fold in length and 8,000-fold in volume, and have different body proportions, with the adult having a relatively smaller abdomen, heavier body armor, and much larger claws. Moreover, the adult and juvenile crayfish have different predators, different defense capabilities, different shelters, and different diets. Given these differences, it is reasonable to expect that the same behavior pattern, LG tailflip, might be used in a similar fashion by adult and juvenile crayfish under some circumstances, and in a different fashion by these animals under other circumstances. At present, however, we currently know too little about the behavioral adaptations of juvenile and adult crayfish to their different niches to understand the different contexts in which this behavior is employed.

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